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Molecular Characterization of Ribonucleic Acid from *Escherichia Coli* Ribosomes

I. Isolation and Molecular Weights

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(*Received 10 February 1960*)

The ribonucleic acid from purified 30 s, 50 s and 70 s ribosomes of *E. coli* has been isolated by the phenol and detergent methods. Both isolation procedures give the same results. The 30 s ribosomes contain 16 s ribonucleic acid, while the 50 s and 70 s particles yield a mixture of 16 s and 23 s ribonucleic acids in varying proportions.

A combination of light scattering and hydrodynamic measurements was used to calculate the molecular weights of the ribonucleic acid. Values of $5{\cdot}6 \times 10^5$ and $1{\cdot}1 \times 10^6$ were obtained for the 16 s and 23 s components, respectively. Since the 30 s ribosome has a molecular weight of approximately 8×10^5 and is 63% ribonucleic acid, it must contain one 16 s unit. The $1{\cdot}8 \times 10^6$ molecular weight 50 s ribosomes also contain 63% ribonucleic acid. It is suggested that they are heterogeneous; some contain one 23 s ribonucleic acid component, while others contain two 16 s units.

1. Introduction

There are three major ribonucleic acid (RNA) components within cells. One is a lower molecular weight (mol wt = 25,000) soluble RNA which participates in the transfer of activated amino acids to the ribonucleoprotein particles (ribosomes), the sites of protein synthesis (Hoagland, Zamecnik & Stephenson, 1957; Berg & Ofengand, 1958; Tissières, 1959). Two larger RNA components with sedimentation coefficients of 15 to 18 s and 23 to 32 s have been found in ribosomes (Timasheff, Brown, Colter & Davies, 1958; Littauer & Eisenberg, 1959; Hall & Doty, 1959). A hint of their origin was suggested when it was realized that the commonly studied ribosomes with sedimentation coefficients of 70 to 80 s are constructed from subunits of unequal size. This is best established for *E. coli* ribosomes, where the two subunit particles (30 s and 50 s) have been isolated, each containing 63% RNA and 37% protein. These aggregate following the addition of Mg^{2+} (or other divalent cations) to form larger particles (Bolten, Hoyer & Ritter, 1958; Tissières, Watson, Schlessinger & Hollingworth, 1959). This situation is illustrated by the scheme:

$$\xrightarrow{\text{increasing } Mg^{2+}}$$

$$\underset{(\text{mol wt} \sim 8 \times 10^5)}{2(30\text{ s})} + \underset{(\text{mol wt} = 1{\cdot}8 \times 10^6)}{2(50\text{ s})} \rightleftharpoons \underset{(\text{mol wt} = 2{\cdot}7 \times 10^6)}{2(70\text{ s})} \rightleftharpoons \underset{(\text{mol wt} = 5{\cdot}4 \times 10^6)}{100\text{ s}}$$

The inequality of the subunit sizes suggests that the 16 s RNA arises from the 30 s particle, while the 50 s ribosome yields 23 s RNA. Here we present data showing this to be the case.

2. Materials and Methods

(a) *Ribosomes*

Ribosomes were isolated from late log phase *E. coli* B cells by the method of Tissières *et al.* (1959). The 30 s, 50 s and 70 s ribosome preparations were at least 90% pure by weight, with the intentional exception of MR 40 (see Fig. 3).

(b) *Apparatus*

A Spinco model L ultracentrifuge was employed for high speed preparative centrifugation. Sedimentation coefficients were measured in a model E Spinco ultracentrifuge, with schlieren and ultraviolet optics. The pictures obtained with the latter were analyzed with a Spinco analytrol and microadapter. The sedimentation coefficients were corrected for viscosity and density to water at 20°C.

Viscosities were measured in an Ostwald-Fenske viscometer with a solvent flow time of 360 sec at 24·98 ± 0·02°C. The partial specific volume of the RNA was measured in a 25 ml. pycnometer. A value of 0·57 ± 0·02 ml./g was obtained in 0·01 M-acetate, 0·1 M-NaCl, pH 4·6 at 24·98 ± 0·02°C.

Optical densities were measured in a Beckman D.U. spectrophotometer. When necessary, this was brought to constant temperature by circulating water from a thermostated bath through the walls of the cell compartment.

Light scattering measurements were made in a Brice-Phoenix photometer. A value of 0·188 for *dn/dc* (Reichman, Rice, Thomas & Doty, 1954) was employed to calculate molecular weights. Both solvent and solutions were freed of dust by repeated filtration through millipore filters (HA, AA) as described by Sinsheimer (1959).

(c) *Chemical methods*

Phosphorus analysis, according to Fiske & Subbarow (Umbreit, Burris & Stauffer, 1957), and the base composition of *E. coli* ribosome RNA (Spahr & Tissières, 1959) were used to calculate the extinction of a 1% RNA solution in 0·01 M-acetate pH 4·6 at 25°C. A value of 227 ± 5 was obtained for $E\,{}^{1\,\mathrm{cm}}_{1\%}$ at 260 mμ.

The protein content of RNA preparations was estimated by a modified biuret analysis (Zamenhof, 1957).

Pancreatic ribonuclease (RNase) was obtained from Worthington Biochemical Corp., Freehold, New Jersey. Cesium chloride purified by treatment with Norite was kindly supplied by David Schlessinger. The sodium dodecyl sulphate (SDS) was washed in cold alcohol and ether to remove ultraviolet absorbing material. Chromatographic, grade 1, neutral alumina was obtained from M. Woelm, Eschwege, Germany.

3. Results

(a) *Preparation of RNA by SDS treatment*

When the ribosomes (suspended in 0·01 M-tris pH 7·4 plus appropriate levels of Mg^{2+}; see Tissières *et al.*, 1959) are exposed to 1% SDS at 37°C for 5 min, they are degraded to their RNA and protein components. The RNA is stable in this incubation mixture, since the latent ribosome RNase (Elson, 1958) is inhibited by 1% SDS. Ribosome protein, like most other protein, has negligible u.v. absorption; as a result, the incubation products can be directly analyzed for their nucleic acid components with the u.v. optics of the analytical ultracentrifuge. The results with the various ribosomes follow:

(1) 70 s ribosomes: two sharp peaks with sedimentation coefficients of 23 s and 16 s are found. They are destroyed by traces of RNase. When this material is centrifuged for 9 hr in a swinging bucket rotor (SW39) at 125,000 *g*, the bottom tenth of the tube contains over 95% RNA. SDS thus effectively separates RNA from most ribosome protein.

(2) 30 s ribosomes: a single sharp 16 s peak is found. It is likewise RNase-sensitive.

(3) 50 s ribosomes: the major peak produced by the purified 50 s particle after SDS treatment has a sedimentation coefficient of 23 s; however, it is always accompanied by a variable amount of a slower moving component (Table 1). As can be seen from Fig. 1(a), this second peak can be quite sharp, and, as such, cannot be considered a random degradation product. However, the slower component may

sometimes be diffuse, as in Fig. 1(b). It is observed that when the slower peak is sharp its sedimentation coefficient is close to 16 S, but when it is diffuse the sedimen-

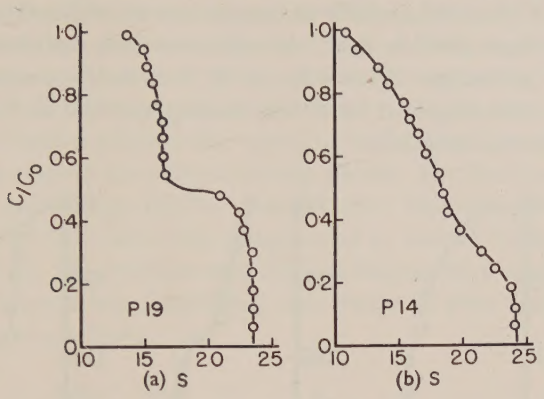

FIG. 1. The integral sedimentation distribution of the RNA from two 50 S ribosome preparations in 0·2% SDS, 0·25 mM-Mg^{2+} and 0·01 M-tris, pH 7·4.

tation coefficient may be smaller or larger. There may be two factors affecting the amount and character of the slower component. First, there appears to be a variation from one particle preparation to another in the amount of the 16 S unit obtained from the 50 S ribosome. In the second place, there is a heterogeneous background, probably due to degradation by traces of nucleases.

TABLE 1

Mean sedimentation coefficients and percentage distribution of RNA components obtained from 50 S *ribosomes with SDS*

Prep.	$S^{I}_{\frac{1}{2}}$‡	$\%S^{I}$†	$S^{II}_{\frac{1}{2}}$§
MR 4	23·4	78	14·3
MR 16	23·0	66	16·5
P 10	24·2	59	19·1
MR 13	23·8	57	17·0
P 5	23·4	56	14·4
P 11	24·0	52	17·5
P 20	23·4	41	16·6
P 19	24·1	36	16·4
P 14	23·3	25	17·2

‡ $S^{I}_{\frac{1}{2}}$ is the mean sedimentation coefficient of the fast component.
† $\%S^{I}$ is the percent of sedimenting material in the fast peak.
§ $S^{II}_{\frac{1}{2}}$ is the sedimentation coefficient of the slow component.

The RNA from the 50 S particle prepared by SDS treatment and centrifugation can be obtained in a pellet with less than 2% protein. When the same material is centrifuged in CsCl at a density of 1·72 g/ml., an RNA pellet is obtained which contains less than 0·3% protein.

(4) Stability of the RNA: the ribonuclease associated with ribosomes has minimum activity at acid pH's (B. Hollingworth & P. F. Spahr, personal communication). Similarly, the RNA obtained by SDS treatment and centrifugation in CsCl was more stable at pH 4·6 than pH 6·8: thus, the sedimentation distribution of this RNA remains virtually unchanged after 4 hr at 37°C in 0·01 M-acetate, pH 4·6, while 30% of the RNA was degraded to slower moving material in 0·01 M-phosphate at pH 6·8 under the same conditions.

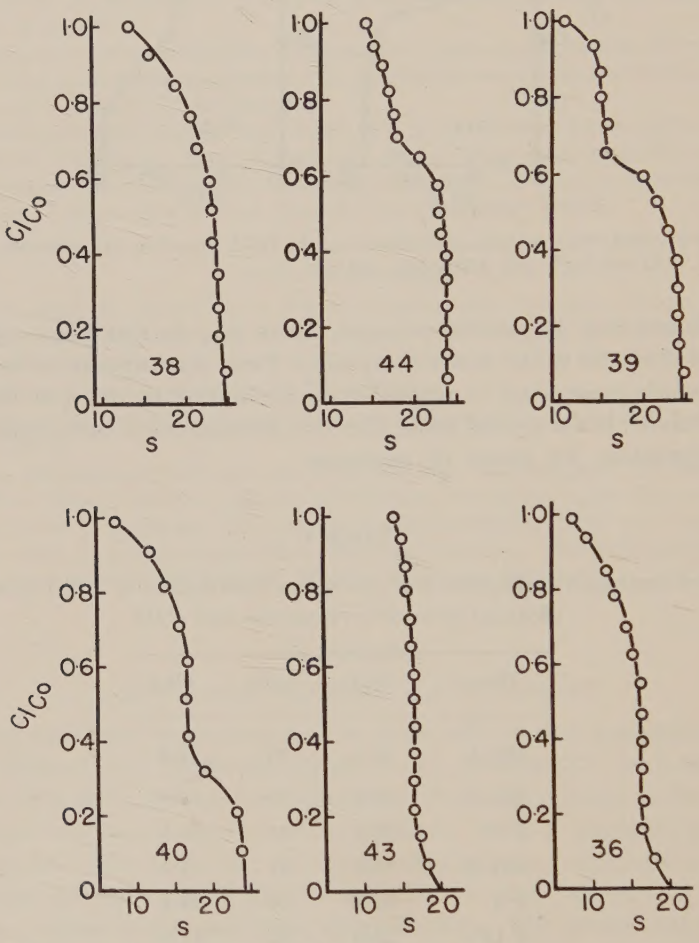

FIG. 2. The integral sedimentation distributions of the RNA from three 50 s and three 30 s ribosome preparations in 0·1 M-NaCl, 0·01 M-acetate pH 4·6. The 30 s ribosome preparation for MR 40 was intentionally prepared with a 30% contamination of 50 s ribosomes.

Heating this RNA, in acetate buffer, for various lengths of time at temperatures ranging from 50 to 90°C randomly degrades the RNA. During this treatment no evidence was obtained to support the notion that either peak can systematically break down into homogeneous subunits with smaller sedimentation coefficients.

(b) *RNA preparation by phenol treatment*

Purified ribosomes suspended in pH 7·4 tris buffer (2-amino-2-hydroxymethyl-propane-1 : 3-diol) and appropriate levels of Mg^{2+} were incubated at room temperature for 5 min with 0·2% SDS. Prior to use, liquified phenol (Merck) was chromatographed

on a column of warm neutral alumina (Woelm) to remove all colored oxidation products. 5 to 20 ml. of a 1 to 3% solution of the SDS-treated particles were shaken with an equal volume of the chromatographed phenol at 30 to 40°C. The mixture was centrifuged for several minutes in a table centrifuge at 1000 rev/min, and the aqueous phase pipetted free of the denser phenol phase (Schramm & Gierer, 1956). This operation was repeated two more times with smaller volumes of phenol.

Two volumes of absolute ethanol were added to the final aqueous phase to precipitate the RNA and thus remove the alcohol-soluble phenol. The flocculent precipitate was centrifuged down and resuspended in acetate buffer. The precipitation was repeated two more times. The final pellet was resuspended in acetate buffer and dialyzed for 6 to 8 hr in the cold. The concentrated RNA solutions were stored at — 25°C. No change in the sedimentation distribution was observed after freezing and thawing the RNA as many as six times.

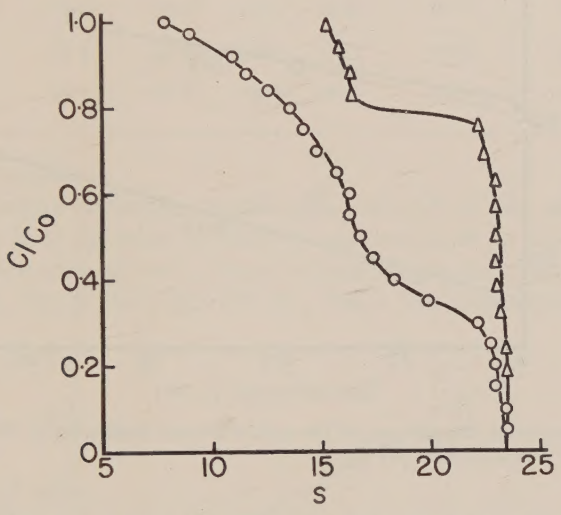

FIG. 3. The integral sedimentation distribution of the RNA from 50 s ribosomes before (△) and after (○) heating for 30 min at 90° C in 0·01 M-phosphate pH 7·0.

The phenol-prepared RNA usually contained less than 1% protein. The recovery of the RNA varied between 70 and 90%; the major loss occurred when the aqueous phase was separated from the phenol phase. The sedimentation coefficients as well as the sedimentation distributions of the RNA prepared by phenol are the same as those obtained with detergent (see Fig. 2).

The RNA prepared by phenol was stable for 30 min at 60°C, while the detergent RNA was not. However, longer incubation times and higher temperatures caused the same pattern of degradation which produced diffuse peaks with the detergent RNA. For example, the integral sedimentation distributions of an RNA preparation from 50 s ribosomes, before and after heating the RNA at 90°C for 30 min, are shown in Fig. 3. This treatment reduces the amount of the 23 s material from 75 to 30%. The remainder of the material is continuously distributed between 8 s and 23 s, with a small peak in the neighbourhood of 16 s. This, taken with the observation that a substantial amount of the 23 s peak remains intact after heating at 90°C, suggests that neither component degrades into homogeneous subunits of the type described by Hall & Doty (1959) for RNA from calf liver ribosomes.

(c) *Physical properties of phenol RNA*

All the values reported below were obtained from the phenol-prepared RNA in 0·1 M-NaCl, 0·01 M-acetate, pH 4·6.

(i) *Sedimentation*

The mean sedimentation coefficient of the RNA obtained from the 30 S ribosome is 16·3 S with extreme variations of ± 0·5 S while that of the main component obtained from the 50 S ribosome is 23·5 S ± 0·7 S.

The sedimentation dependence on concentration is plotted in Fig. 4. The values for the slope of this plot are 0·007 S^{-1}/g/dl. for the 16 S peak and 0·011 S^{-1}/g/dl. for the 23 S peak. Therefore, at the concentrations used for the u.v. optics of the analytical centrifuge (40 μg/ml.) the concentration effect is negligible.

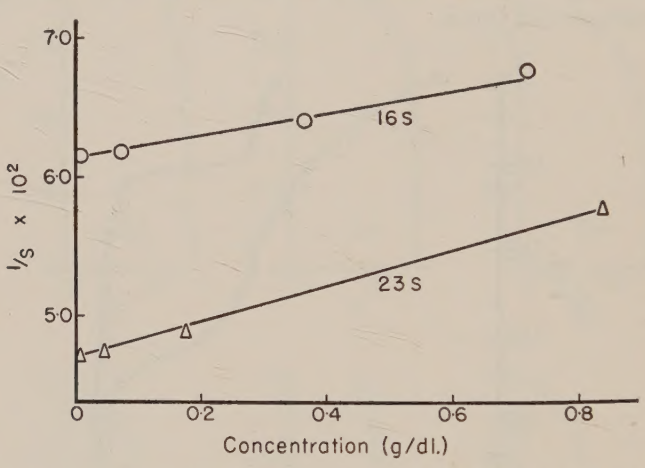

FIG. 4. The concentration dependence of the sedimentation coefficient of 16 S (○) and 23 S (△) RNA in 0·1 M-NaCl, 0·01 M-acetate pH 4·6.

This means that the only necessary correction that must be applied to the densitometer tracings of the sedimenting boundaries is that for radial dilution. If the densitometer tracing of the sedimenting boundary is divided into ten or more sections and the mean sedimentation coefficient of each is calculated, then, after the radial dilution correction is applied (Shooter & Butler, 1956), the average of these values per unit concentration will give a weight average sedimentation coefficient (S_w). That this weight average sedimentation coefficient gives a more meaningful index of the degree of heterogeneity of the RNA than the mean sedimentation coefficient is clear from a consideration of Fig. 2. The values of S_w for six preparations are listed in Table 2.

(ii) *Viscosity*

The intrinsic viscosity of six RNA preparations is listed in Table 2. These values were obtained by plotting three values of η_{sp}/c as a function of the concentration (c) for each preparation. The viscosity dependence on concentration was found to obey the Huggins' relationship (Huggins 1942):

$$\frac{\eta_{sp}}{c} = [\eta] + k'[\eta]^2 c \qquad (1)$$

While the intrinsic viscosity of the three preparations from 30 S ribosomes are close to 0·3, those found for the preparations from 50 S ribosomes are more widely dispersed

about the value of 0·4. This variation in the latter group reflects their greater variation in polydispersity.

TABLE 2

Measured physical properties of ribosome RNA

Prep.	Ribosome source	S_w	$[\eta]$	k'	$M_w \times 10^{-5}$	$\beta \times 10^{-6}$
36	30 s	14·5	0·272	0·98	4·60	2·21
40	30 s	16·7	0·308	0·92	5·98	2·22
43	30 s	16·1	0·298	0·87	5·20	2·27
38	50 s	21·5	0·461	0·99	9·11	2·32
39	50 s	19·8	0·360	0·81	8·40	2·22
44	50 s	20·9	0·394	0·77	8·85	2·28

(iii) *Molecular weights*

The weight average molecular weight (M_w) of each of the preparations whose S_w and $[\eta]$ have been recorded in Table 2 was measured by light scattering. The measurements were made at two or three concentrations and plotted in a Zimm plot (Zimm, 1948) to obtain the value for M_w. Since the preparations used were not

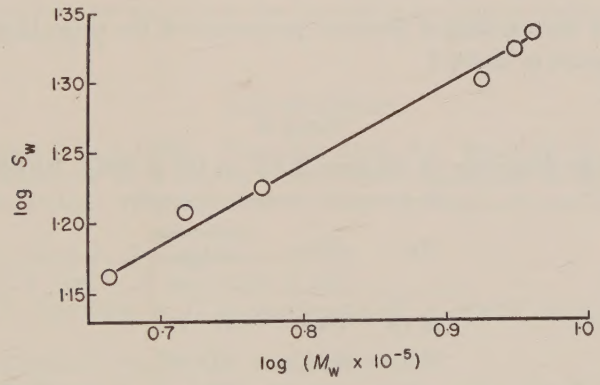

FIG. 5. The molecular weight dependence of the sedimentation coefficient in 0·1 M-NaCl, 0·01 M-acetate pH 4·6.

monodisperse, an indirect calculation is used to obtain the molecular weights of the 23 s and the 16 s peaks. This can be done in two ways. If the values of log S_w are plotted against the log M_w (Fig. 5) an empirical equation is obtained:

$$S_w = 0{\cdot}98 \times 10^{-2} M_w^{0{\cdot}56} \qquad (2)$$

The molecular weights obtained from (2) for the 16 s and 23 s peaks are $5{\cdot}6 \times 10^5$ and $1{\cdot}1 \times 10^6$ respectively, with an error of $\pm$ 12%.

Previous work (Hall & Doty, 1959) shows that RNA behaves hydrodynamically as an ellipsoid of revolution. The Scheraga & Mandelkern (1953) equation (3) is thus applicable, and the data in Table 2 have been used to calculate the shape-volume coefficient β. An average value of $2{\cdot}25 \times 10^6$ is obtained for β. In this calculation

S_w corrected to 25°C is used for S, η_0 is the viscosity of solvent, ρ is the solvent density, N is Avogadro's number and $\bar{v}$ is the partial specific volume.

$$M_w^{2/3} = \frac{S[\eta]^{1/3}\,\eta_0\,N}{\beta(1-\bar{v}\rho)} \tag{3}$$

Then, by plotting $\log S_w$ *vs.* $\log [\eta]$ (Fig. 6) values of 0·445 for the intrinsic viscosity of the 23 s peak and 0·305 for the 16 s peak are obtained. Putting these values back into (3), molecular weights of $1{\cdot}15\times10^6$ and $5{\cdot}6\times10^5$ are obtained with a maximum error of $\pm$ 15%.

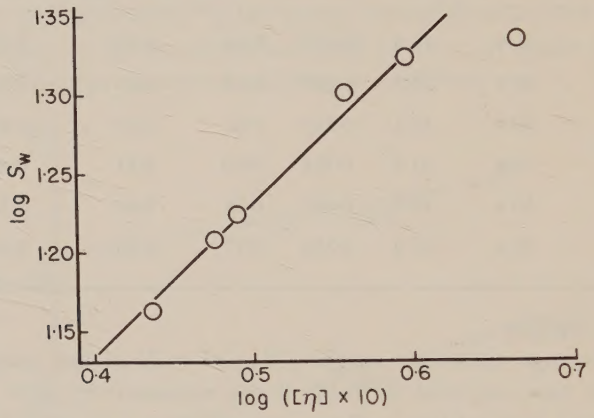

FIG. 6. A plot of sedimentation coefficient *vs.* intrinsic viscosity in 0·1 M-NaCl, 0·01 M-acetate, pH 4·6.

A summary of the calculated physical properties of the pure 16 s and 23 s components is presented in Table 3.

TABLE 3

Calculated physical properties of ribosome RNA in 0·1 M-*NaCl, 0·01* M-*acetate, pH 4·6*

S_{20}	$[\eta]$	molecular weight
16·3 s	0·305	$5{\cdot}6\times10^5$
23·5 s	0·445	$1{\cdot}1\times10^6$

4. Discussion

The 30 s ribosome is nearly two-thirds RNA and has a molecular weight of approximately 8×10^5 (Tissières *et al.*, 1959). When the RNA is isolated from this particle by detergent or phenol treatment, a single 16 s component is obtained. Clearly, the 30 s particle contains a single $5{\cdot}6\times10^5$ molecular weight RNA component.

The RNA of the 50 s ribosome is more complex; thus, both detergent and phenol preparations show a mixture of two components with sedimentation coefficients of 16 s and 23 s. The slower component could be a structurally different form of the 23 s RNA, rather than a lower molecular weight component. However, the systematic dependence of M_w on S_w (Fig. 4) argues against this conclusion. Since the 50 s particle ($M_w = 1{\cdot}8\times10^6$) likewise has nearly two-thirds RNA (Tissières *et al.*, 1959),

the maximum molecular weight of RNA in a 50 s particle is $1{\cdot}1 \times 10^6$. Therefore, the 50 s particle fraction must be heterogeneous: some of the 50 s particles contain one 23 s component of molecular weight $1{\cdot}1 \times 10^6$, while others contain two 16 s units with molecular weights of $5{\cdot}6 \times 10^5$.

Since the molecular weight ratio of the 23 s to the 16 s RNA is 2 : 1, it is likely that the weight ratio of the 50 s particle to the 30 s particle is the same. Hence, the 50 s particle could be formed by the fusion of two 30 s particles, and those 50 s particles containing two 16 s RNA units might represent intermediates in this process. Such an hypothesis is consistent with the observation of Spahr & Tissières (1959), who report that the nucleotide composition of the RNA from the 30 s particle is the same as that from the 50 s particle.

Heating the RNA or incubating with 8 M-urea does not form smaller homogeneous peaks. Therefore, if the 23 s component is formed by the fusion of two 16 s units, this must be accomplished by the formation of a covalent bond. On the other hand, the combination of a 30 s particle with a 50 s particle to form a 70 s ribosome does not lead to the formation of primary bonds between their respective RNA components, since no RNA peak larger than 23 s was isolated from the 70 s particles.

The guidance of Professor J. D. Watson is gratefully acknowledged. The author wishes to thank Professor P. M. Doty for several helpful discussions and the use of his light scattering equipment. Thanks are due Drs. H. Boedtker-Doty and J. Eigner for valuable instruction.

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Hypochromicity in Ribosomes from *Escherichia Coli*

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(*Received 10 February 1960*)

The hypochromicities of *E. coli* ribosomes and of their isolated RNA, measured by KOH and ribonuclease hydrolysis, are identical. It is suggested that the RNA configuration in solution is retained in ribosomes, and may be a well-defined structure with biological implications.

1. Introduction

Recent studies of ribonucleic acid (RNA) in salt solutions suggest that hydrogen-bonded regions, helically arranged, alternate with non-hydrogen-bonded regions (Doty, Boedtker, Fresco, Haselkorn & Litt, 1959). The hydrogen-bonded segments may be part of a well-defined structure with biological significance. However, the possibility remained that the structure in solution bears no relation to the RNA configuration within ribonucleoprotein particles (ribosomes), the probable site of protein synthesis.

Strong evidence for hydrogen bonding in polynucleotides is their hypochromicity. Polynucleotides absorb less ultraviolet light than equivalent mixtures of mononucleotides, and as shown for deoxyribonucleic acid (DNA) and synthetic polyribonucleotides of known configuration, there are two contributions to this hypochromicity. When nucleotides are assembled in a chain, the extinction is lowered by about 10%. If the assembled chain takes up an ordered configuration stabilized by hydrogen bonds, a further lowering of 30 to 60% takes place. All hypochromicity, except the contribution from chain formation, is abolished when secondary ordering is destroyed, for example by heating. For a given environment, each hydrogen-bonded polynucleotide is characterized by a melting temperature, at which half the hydrogen-bonded hypochromicity is abolished (Doty, Boedtker, Fresco, Hall & Haselkorn, 1959). RNA, in contrast to DNA, shows lower and less sharp melting behaviour, interpreted by the postulate of a less ordered structure. Addition of 8 M-urea or other hydrogen-bonding reagents greatly lowers melting temperatures and in some cases abolishes all configurational hypochromicity.

Recently Hall & Doty (1959) demonstrated that rat liver ribosomes also show a hypochromic effect, and concluded that RNA bound in ribosomes, like free RNA, is partially hydrogen bonded. Here we present data which show that the hypochromicities of *Escherichia coli* ribosomes and isolated ribosomal RNA are the same.

2. Materials

30 s, 50 s, and 70 s ribosomes of *E. coli*, containing 63% RNA and 37% protein, were isolated by differential centrifugation (Tissières, Watson, Schlessinger & Hollingworth, 1959). Purified RNA from 30 s, 50 s, and 70 s ribosomes was obtained in highly poly-

merized form from Kurland (1960) and dialysed for 12 hr against 500 vols 0·01 M-phosphate buffer, pH 7·0 + 0·001 M-magnesium acetate, before use. Southern bean virus was obtained from Dr. Russell Steere. Bushy stunt virus and turnip yellow mosaic virus were provided by Dr. Donald Caspar.

Reagent grade NaCl, KOH, and Fisher Standard N-HCl were used. Urea was recrystallized from distilled water until its optical density in 8 M-solution was below 0·150 at 260 μ. The buffer used was 0·01 M-phosphate, pH 7·0 + 0·001 M-magnesium acetate.

A model L preparative Spinco ultracentrifuge was used. Centrifugal forces are expressed as ***g*** at the middle of the centrifuge tube.

A Beckman DU spectrophotometer was used. In experiments measuring the variation of ultraviolet absorption with temperature, the solution temperature was controlled by circulating water from a thermostated bath through coils in the walls of the cell compartment.

3. Results

(a) *Hypochromicity of E. coli RNA*

Purified *E. coli* RNA from several particle fractions shows a total hypochromic effect of 41 $\pm$ 2% (Table 1). Digestion of the polynucleotides to mononucleotides

TABLE 1

Percentage increase of optical density at 260 mμ

Sample	Treatment: KOH hydrolysis	*E. coli* ribonuclease	heating to 85°C	8 M-urea	saturated NaCl
	%	%	%	%	%
30 s RNA	40	41	29	26	0
50 s RNA	41	42	29	27	0
70 s RNA	40	43	29	24	0
30 s ribosome	42	—	—	27	0
50 s ribosome	41	—	—	25	0
70 s ribosome	42	—	—	25	0

was achieved in two ways. First the material was hydrolysed with 0·24 N-KOH for 18 hr, to give a mixture of 2′ and 3′ nucleotides. Table 1 gives the percentage increase in optical density at 260 mμ, after the hydrolysate was brought to pH 7·0 with 0·3 N-HCl. All values are the average of at least four hydrolyses, with a maximum deviation of 4%. A second measure of total hypochromicity was obtained by digestion of RNA with purified *E. coli* ribonuclease (Elson, 1958). This enzyme specifically digests RNA to nucleoside 3′-phosphates (P. F. Spahr, personal communication). The hypochromicity contributed by secondary ordering into helical regions was estimated by heating RNA samples and measuring the optical density as a function of temperature. The melting temperature of all samples was about 60°C and Table 1 lists the maximum percentage increases. Nearly all the increase was reversible when the temperature was lowered to 25°C. Urea also breaks hydrogen bonds and addition of 8 M-urea at 20°C raises the 260 mμ absorption to values approaching those found after heating to 85°C (Table 1). On the contrary, high salt concentrations stabilize helical regions in polynucleotides, and no effect was anticipated or found after addition of saturated NaCl.

(b) *Hypochromicity of E. coli ribosomes*

There is a 42% increase of specific absorption at 260 mμ when the various particle fractions, treated with 0·24 N-KOH for 18 hr, are brought to pH 7·0 with 0·3 N-HCl. Intact particles show Rayleigh scattering; its magnitude was determined by measuring scattering *versus* wavelength and extrapolating into the region of consumptive absorption (Bayzer & Schauenstein, 1955). Scattering contributes 3% of the optical density of a 70 s ribosome solution at 260 mμ, and in Table 1 all values are so corrected.

The absorption increase results from RNA hydrolysis, and not from any scattering by ribosomal protein coagulated during treatment with base. Thus, after neutralized samples are centrifuged for 2 hr in the SW 39 swinging bucket rotor at 110,000 ***g***, the top 3 ml., carefully pipetted off, show less than a 3% decrease in 260 mμ absorption in comparison to the uncentrifuged control.

Heating ribosomes and measuring ultraviolet absorption at 260 mμ does not give the hypochromicity contributed by secondary structure. When ribosomes are heated

TABLE 2

Hypochromicity in RNA viruses

Virus	% RNA	% protein	% increase in 260 mμ absorption after KOH hydrolysis
Bushy stunt	16	84	47
Turnip yellow mosaic	37	63	47
Southern bean mosaic	21	79	56

there is less than a 2% increase in absorption until about 55°C. At this temperature, the particles break down releasing a latent ribonuclease (Elson, 1958), which digests the ribosome RNA to nucleotides. Addition of 8 M-urea immediately breaks down ribosomes to their protein and RNA components. No ribonuclease action occurs immediately, however, since it is inhibited by the high urea concentration. Here the initial optical density rise is 25%, the amount expected by destruction of secondary structure in the RNA component.

(c) *Hypochromicity in RNA viruses*

A number of small viruses, like ribosomes, contain only RNA and protein as major components (Markham, 1959). The hypochromicity of several of these, turnip yellow mosaic virus, bushy stunt virus, and southern bean mosaic virus, was measured after hydrolysis with 0·24 N-KOH for 18 hr and subsequent neutralization with 0·3 N-HCl. All showed (Table 2) increases in absorption at 260 mμ of 47 to 56% after hydrolysis, that is, equal or greater than observed with *E. coli* ribosomes. These values were corrected for Rayleigh scattering by extrapolation from values at longer wavelengths.

4. Discussion

The identical hypochromicity of *E. coli* ribosomes and their isolated RNA argues that the RNA configuration in solution is basically retained after its packaging into ribosomes. This strengthens the possibility that this configuration does not represent a haphazard collection of randomly formed hydrogen bonds, but may be a well-defined structure whose eventual elucidation has important biological implications.

The marked hypochromicity observed for the several spherical RNA viruses hints that hydrogen-bonded regions also occur in their RNA component. This need not, however, be an absolute requirement for RNA structure, since in the rod-shaped tobacco mosaic virus the single RNA chain is helically arranged in a way which prohibits hydrogen bonding between bases (Franklin, Caspar & Klug, 1959).

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Genetic Determination and Enzymatic Induction of Tyrosinase in *Neurospora* † ‡

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Three genes controlling the synthesis of tyrosinase in *Neurospora crassa* have been found: *T*, *ty*-1, and *ty*-2. The genes are not closely linked. Of the three, only the *T* gene has a structure-determining role in the synthesis of the enzyme. The other two influence the synthesis indirectly through their control over an inductive mechanism that operates on the enzyme. Aromatic amino acids, especially their D-stereoisomers, are effective as inducers. Sulfate, or a substance derived from it, acts as a repressor of the synthesis. Tyrosinase formation in wild-type is constitutive or inductive, depending on the concentration of sulfate in the medium.

1. Introduction

Previous publications have described two forms of tyrosinase, differing in thermostability, in wild-type strains of *Neurospora crassa* (Horowitz & Fling, 1953, 1956*a*). The thermostability difference was found to be simply inherited, the stable (*S*) form of the enzyme being determined by a gene, T^S, and the labile (*L*) form by its allele, T^L. The two enzymes also differ electrophoretically, the *L*-form migrating cathodically ahead of the *S*-form on paper at pH 6.‖ Various lines of evidence, including the simultaneous production of both forms in heterocaryons, show that the observed thermostability and electrophoretic differences reflect a structural dissimilarity between the enzymes. The *T* locus may therefore be presumed to have a structure-determining role in the synthesis of tyrosinase (Horowitz & Fling, 1956*b*).

The present paper deals with two mutants that, under the usual conditions of growth, are deficient in tyrosinase. It has been found that these mutations are not allelic with or closely linked to the *T* locus or each other. The role of these two genes in the synthesis of tyrosinase has been investigated. It will be shown that they do not take part in determining the structure of the enzyme, but influence the level of tyrosinase activity indirectly through their control over an inductive mechanism that has been found to operate on the enzyme.

2. Materials and Methods

(a) *Strains*

The mutants to be described—called tyrosinaseless-1 (*ty*-1) and tyrosinaseless-2 (*ty*-2), respectively—were kindly made available to us by Prof. M. Westergaard. They originated

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‖ Further details of the electrophoretic results will be given in a separate publication.

from the collection in Baarn, Holland, and are presumed to be spontaneous mutants. They were identified as tyrosinase-deficient by Prof. Westergaard, who also found that they are female-sterile. The female sterility is expressed in the failure to form protoperithecia on crossing medium. They are male-fertile, however, and they grow like wild-type on liquid minimal medium. On solid media, *ty*-1 shows a visible character designated "velvet"—i.e. the mycelium and conidia are produced close to the surface of the medium, without the usual profusion of aerial hyphae. *ty*-2 is morphologically normal. Neither female sterility nor "velvet" has been observed to separate from the tyrosinase deficiency in crosses. The original strain of *ty*-1 and its genetic derivatives usually produce no, or only traces of, tyrosinase; occasionally they show low, but significant, activity. *ty*-2 and its derivatives are frequently devoid of activity also, but they tend, more than does *ty*-1, to form small amounts of the enzyme. Both mutants differ in two important respects from the (possibly phenotypic) tyrosinase-deficient variants previously found by Horowitz & Fling (1953) and by Fox & Burnett (1959): they segregate regularly, and, unlike some of the variants mentioned, they show no increased tyrosinase production on prolonged incubation.

(b) *Methods*

Cultures were grown for 4 days at 25° C on a modified Fries medium ("low-S" medium) containing 0·06 mM-sulfate (Horowitz & Shen, 1952), or on Vogel's medium N (Vogel, 1956) modified to contain this amount of sulfate. For certain experiments a "high-S" medium, containing 8 times as much sulfate, was employed. Routine assays of tyrosinase activity were carried out in crude extracts in 0·1 M-sodium phosphate buffer, pH 6. Where it was desired to obtain an accurate measure of thermostability, as in the heterocaryon experiments, the enzyme was purified by the previously described procedure (Horowitz & Fling, 1953). Thermostability tests were carried out at 59 $\pm$ 0·02°C in 0·1 M-sodium phosphate, pH 6.

The assay system for the enzyme consisted of 0·1 ml. of extract or purified enzyme preparation, 3·9 ml. of 0·1 M-sodium phosphate buffer, pH 6, and 4 mg of 3,4-dihydroxy-DL-phenylalanine (DL-dopa) added in 1 ml. of the same buffer. The formation of dopachrome at 30°C was followed either colorimetrically with the Klett-Summerson photoelectric colorimeter, using filter No. 42, or spectrophotometrically at 475 mμ (Mason, 1948). A *tyrosinase unit* is defined as the calculated amount of enzyme that, under these conditions, will cause an increase in optical density of 1 unit per cm of light-path (equivalent to 500 colorimeter units) in the first 5 min after mixing.

For paper electrophoresis, a Spinco (Durrum type) apparatus was used in a room kept at 5 to 10°C. The paper strips were pretreated with a 1% solution of bovine serum albumin† in 0·05 M-sodium phosphate, pH 6, to facilitate migration of the enzyme. The same buffer, containing 0·1% bovine serum albumin, was used in the reservoirs. Between 0·02 and 0·06 units of enzyme were applied to each strip. The current was 1·25 mA per strip for 16 to 24 hr. The enzyme was visualized at the end of the run by spraying the strips with a 0·02 M-solution of DL-dopa buffered at pH 6.

3. Genetics

The tyrosinase-deficient mutants were crossed to wild-type strains carrying T^L, and tetrads were isolated. Cultures grown on low-S medium were assayed for tyrosinase activity, and the form of the enzyme (S or L) was determined in those cultures which produced significant enzyme activity. In the case of *ty*-1, a second generation was obtained by crossing some of the tyrosinaseless progeny from the first generation to T^S or T^L wild-types. The results are summarized in Fig. 1.

The segregations are consistent with single gene inheritance of *ty*-1 and *ty*-2—i.e. each tetrad contained two tyrosinase-deficient and two normal spores—but the data on this point are not extensive. More important for the present purpose is the finding

† Armour Laboratories Fraction V.

that neither mutant is allelic with the *T* locus. Non-allelism is proved by the appearance of T^S in the first generation tetrads, and this is confirmed, for *ty*-1, by the second generation tetrads. Since the wild-type parent carried T^L in both first generation crosses, it follows that T^S was present, but not expressed, in the mutants. The extensive recombination that is evident between the characters (tyrosinase production *vs.* the form of the enzyme) in both pedigrees indicates non-linkage of *ty*-1 with *T* and, at best, loose linkage between *ty*-2 and *T*.

Allelism between *ty*-1 and *ty*-2 could not be tested by the simple cross *ty*-1 × *ty*-2, owing to the female sterility of both mutants. The cross could be effected by the use

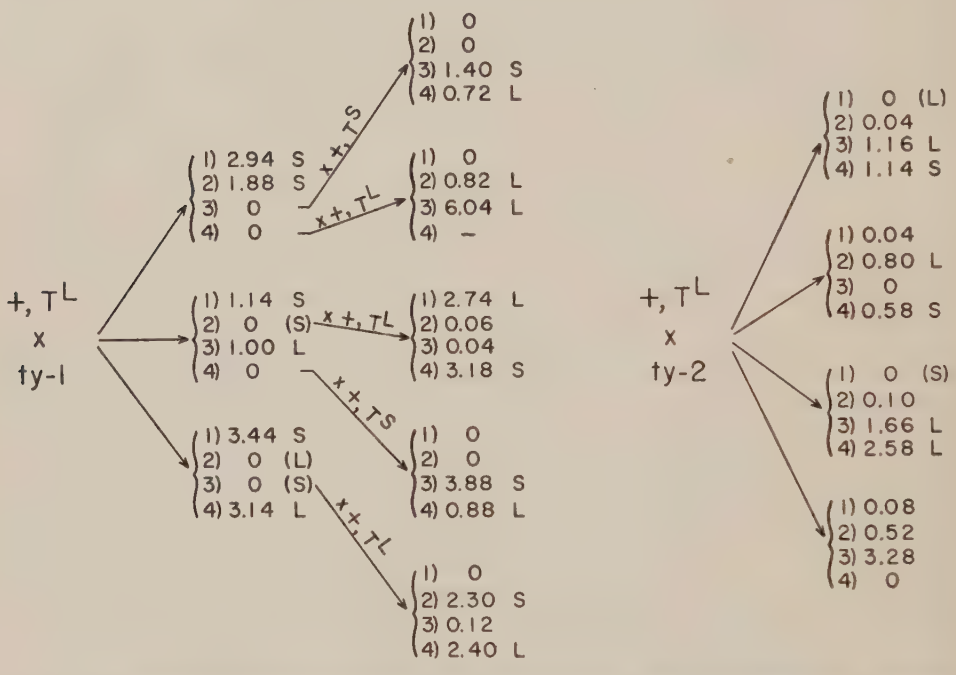

FIG. 1. Pedigrees showing non-allelism of the *T*-locus with *ty*-1 and *ty*-2, respectively. Separate tetrads are in braces, with their (ordered) spore pairs numbered 1 to 4. The figures are tyrosinase activities in tyrosinase units per ml. of extract, each ml. containing the enzyme from 0·25 to 0·5 g (wet weight) of mycelium. Activities exceeding 0·5 are in the wild-type range. Letters indicate the form of the enzyme produced. Letters in parentheses indicate that the form of the enzyme was determined in induced cultures.

of a heterocaryon, however, since the mutant characters are recessive to normal. A heterocaryon of the following constitution was made up:

$$(A, +, \textit{hist}\text{-}3, T^L; \textit{ty}\text{-}1)\ (A, \textit{ad}\text{-}3, +, T^S; +),$$

where *A* refers to mating-type and the parentheses enclose the genotypes of the two kinds of nuclei making up the heterocaryon. The biochemical markers histidineless-3 and adenineless-3 were used to balance the heterocaryon, in the usual way. The heterocaryon is fully fertile, is non-velvet in morphology, and produces tyrosinase. It was used as the protoperithecial (i.e. female) parent in a cross to a strain of the constitution *a*, T^S; *ty*-2. Since tetrads segregating for the histidineless marker must have originated from a cross between the two different tyrosinaseless nuclei, a number of these were isolated in order to determine whether recombination between *ty*-1 and *ty*-2 had occurred. Cultures were grown from the spores, and the level of tyrosinase

activity was determined in the non-velvet, presumably *ty*-1 +, cultures in each tetrad. The results are shown in Table 1. The finding of normal (i.e. wild-type) tyrosinase

TABLE 1

Tyrosinase activities of the non-velvet progeny in tetrads showing segration of the hist-3 marker, from the cross

Het (*A*, +, *hist*-3, T^L; *ty*-1) (*A*, *ad*-3, +, T^S; +) × *a*, T^S; *ty*-2†

Tetrad	1	2	3	4	5	6	7	8
Spore pair								
1	+ *vt*	+ 0·00	+ *vt*	+ *vt*	*hist vt*	+ 0·00	+ 0·28	+ *vt*
2	*hist vt*	+ 7·02	*hist* 0·08	*hist vt*	+ 2·55	+ 2·66	+ *vt*	+ 12·8
3	+ 0·04	*hist vt*	*hist* 0·00	+ 2·16	+ *vt*	*hist vt*	*hist* 0·00	*hist* 0·00
4	*hist* 0·00	*hist vt*	+ *vt*	*hist* 1·44	*hist* 0·00	*hist vt*	*hist vt*	*hist vt*

† Tyrosinase activities in tyrosinase units per g (wet weight) of mycelium. Values exceeding 1·00 are in the normal range of activity. *vt* = "velvet".

values in some of the progeny shows that recombination between *ty*-1 and *ty*-2 occurred, and these genes are therefore not alleles. The amount of recombination was considerable, as shown by the fact that of the four tetrads (nos. 1, 3, 4, and 7) that are ditypes with respect to *ty*-1 and *ty*-2, one (no. 4) is a non-parental ditype. This is an indication that the two genes are not linked.

4. Further Studies on Heterocaryons

In order to determine the dominance relations of *ty*-1 and *ty*-2 and to study their effect on the form of the tyrosinase produced, the following heterocaryons were constituted:

heterocaryon A: (*a*, +, *hist*-3, T^S; *ty*-1) (*a*, *ad*-3, +, T^S; +)
heterocaryon B: (*A*, +, *hist*-3, T^L; *ty*-1) (*A*, *ad*-3, +, T^S; +)
heterocaryon C: (*A*, +, *hist*-3, T^L; *ty*-1; +) (*A*, *ad*-3, +, T^S; +; *ty*-2).

All of the heterocaryons produced tyrosinase in the normal range of activities. *ty*-1 and *ty*-2 are therefore recessive to their normal alleles. The form of the enzyme produced by the heterocaryons corresponded in every case to the *T*-alleles present. Thus, heterocaryon A produced the *S* form only, while heterocaryons B and C produced both *S* and *L*. This is shown in the heat inactivation curves of Fig. 2. The heat inactivation results were confirmed by paper-strip electrophoresis of the enzyme preparations.

5. Induction of Tyrosinase

(a) *Induction in the mutants*

Various unsuccessful attempts to analyse the biochemical defect of the mutants were made. Thus, addition of mutant extracts to an active enzyme preparation, or the mixing of extracts of *ty*-1 and *ty*-2, failed to show the presence of a tyrosinase inhibitor or the lack of an activator in the extracts. Fractionation of the extracts by a

procedure that leads to an 800-fold purification of the enzyme from wild-type failed to yield any activity from either mutant. Attempts to demonstrate a serologically cross-reacting material in mutant extracts by the use of a rabbit antiserum† active against both *S* and *L* forms of the enzyme likewise gave negative results. Then it was found that both mutants can be *induced* to form tyrosinase by adding an aromatic amino acid to the medium (Table 2). Enzyme activities equal to or exceeding

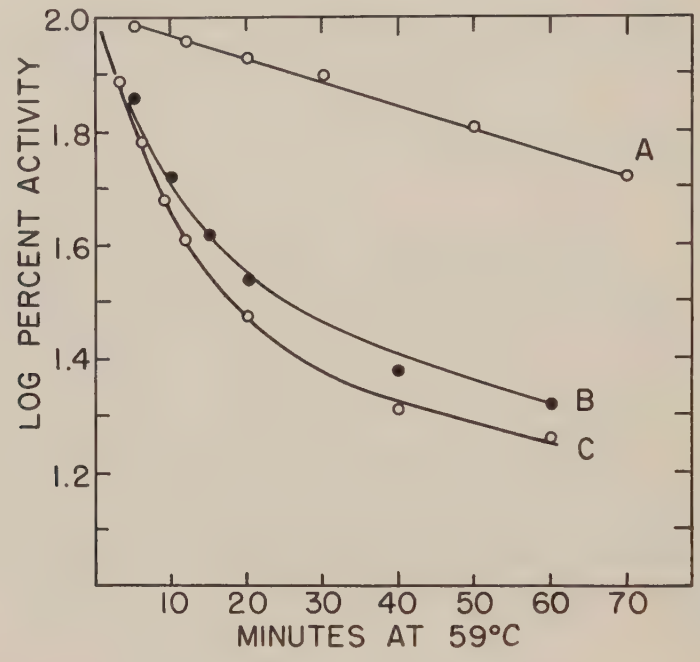

FIG. 2. Thermal inactivation of tyrosinase from the 3 heterocaryons described in the text. Points are experimental values; curves are theoretical for the following mixtures: het. A, 100% of half-life 72 min; het. B, 40% of half-life 65 min + 60% of half-life 5 min; het. C, 32% of half-life 70 min + 68% of half-life 5 min. The variation in the half-life of the *S*-form is due to impurities in the preparations.

those found in wild-type strains are attained. It is not required for induction that growth occur in the presence of the inducer, since similar results are obtained with resting mycelium in phosphate buffer to which an inducer has been added. If a transfer is made from an induced culture to fresh medium free of inducer, the mycelium that grows up is devoid of tyrosinase activity.

The induction is characterized by a number of interesting features as regards specificity, concentration dependence, and repression. Effective as inducers are tyrosine, dopa, phenylalanine, and tryptophan. The first two are substrates and the last two are competitive inhibitors of tyrosinase. Surprisingly, the D-stereoisomer‡ is in every case far more active than the corresponding L-stereoisomer. The significance of the stereoisomeric preference is not clear at this time. Both enantiomorphs of tyrosine and dopa are oxidized by the enzyme, and both enantiomorphs of phenylalanine and tryptophan are inhibitory.

† We are indebted to Prof. Ray Owen for preparing the antiserum and instructing us in its use.

‡ The D-stereoisomers of tyrosine, tryptophan, and phenylalanine were the gift of Dr. Milton Winitz, National Cancer Institute, N.I.H., to whom we express our thanks.

The following substances failed to induce when added to growing cultures of *ty*-1: tyramine, 3-amino-L-tyrosine, prephenate, *p*-aminobenzoate, phenylpyruvate, DL-phenylglycine, phenylacetate, *p*-hydroxyphenylacetate, catechol, DL-alanine, and DL-glutamate.

TABLE 2

Induction of tyrosinase activity in mutant and wild-type Neurospora †

Strain	Medium	Inducer	Inducer concentration (mg/ml.)	Stereoisomer (where applicable) D-	L-	DL-
ty-1	low-S	none	—	0·0	—	—
		tyrosine	0·25	47·2	0·30	47·6
		phenylalanine	0·25	74·4	3·85	75·4
		tryptophan	0·25	101·6	1·44	39·6
		dopa	0·25	28·7	1·13	—
ty-2	low-S	none	—	0·00	—	
		dopa	0·074	4·36	0·00	
wild-type 69-1113a	low-S	none	—	10·85	—	
	high-S	none	—	1·38	—	
		tryptophan	0·37	14·7	1·34	
		dopa	0·37	37·0	1·93	

† Cultures were grown for 4 days on low-S or high-S medium, as indicated, containing the inducer. Values under "Stereoisomer" are tyrosinase units per g (wet weight) of mycelium.

In Fig. 3 are plotted induced tyrosinase activities as a function of the concentration of inducer, for several inducers. It appears that a threshold concentration may exist for the L-stereoisomers, below which no significant induction occurs. There is also the possibility of a threshold for induction by D-stereoisomers, but at much lower concentrations.

The form of the enzyme produced following induction was determined on a number of different genetic derivatives of the original mutants. It was found that some of the derived strains (of both mutants) produced *S*-tyrosinase and others *L*-tyrosinase. In other words, the genes *ty*-1 and *ty*-2 do not influence the properties of the induced enzyme. The *T*-locus is evidently responsible for determining the structure of induced, as well as constitutive, tyrosinase. This conclusion was confirmed for several derived *ty*-1 and *ty*-2 strains by independent genetic evidence showing that they carried the *T*-allele corresponding to the inductively formed tyrosinase (see Fig. 1).

It is interesting to note that the female sterility of the mutants was not cured by the incorporation of inducers in the crossing medium. The possibility that induction does not take place on crossing medium seems excluded by the fact that blackening of the mycelium (presumably due to melanin production) occurred when inducers were present. Wild-type produced protoperithecia abundantly on the same media. The velvet morphology of *ty*-1 is also unchanged in induced cultures.

(b) *Induction in wild-type*

It was of considerable interest to find out whether induction of the enzyme is limited to certain mutants, or whether it can also be demonstrated in wild-type

Neurospora. As we have previously noted (Horowitz & Shen, 1952), tyrosinase production, which is constitutive in wild-type growing on low-S medium, is apparently inhibited on sulfate-rich media. We have now found that wild-type exhibits an inductive response when grown on a medium which contains enough sulfate to inhibit

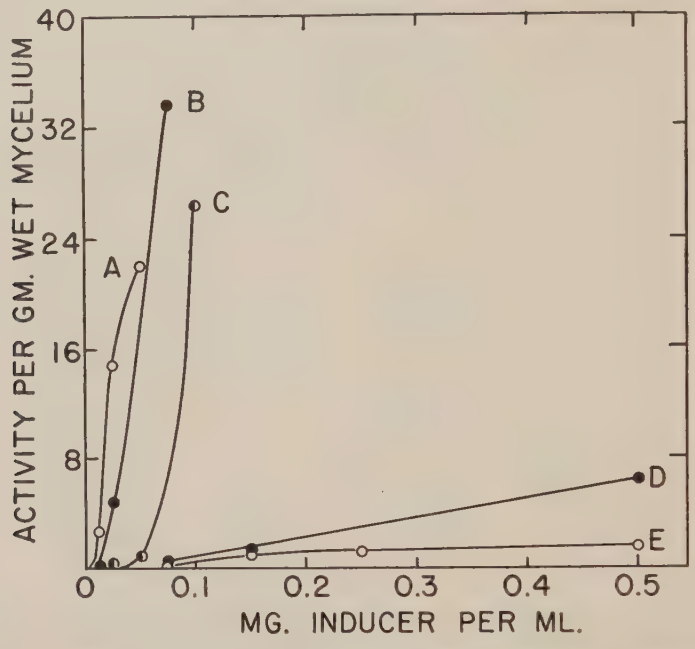

FIG. 3. Induction of tyrosinase activity in *ty*-1 as a function of inducer concentration. Cultures were grown for 4 days in the presence of the following: curve A, DL-tryptophan; curve B, D-dopa; curve C, DL-phenylalanine; curve D, L-tryptophan; curve E, L-phenylalanine. Activity in tyrosinase units.

constitutive synthesis. This is shown in Table 2. Sulfate, or a substance derived from it, behaves like a repressor (Vogel, 1957) of tyrosinase synthesis, although it is not a product of tyrosinase activity. The induction in wild-type appears to be similar in its general characteristics to that found in the mutants.

6. Discussion

The results which have been presented show that tyrosinase production in *Neurospora* is under the control of at least three genes. Of the three known loci, only one—the *T* locus—appears to have a structure-determining role in the synthesis of the tyrosinase molecule. The other two, identified by their recessive mutants *ty*-1 and *ty*-2, do not affect the structure of the enzyme insofar as this is reflected in thermostability and electrophoretic properties. The role of these two genes in tyrosinase synthesis seems to be an indirect one, since the induction experiments show that both tyrosinaseless mutants possess the essential genetic mechanism for production of the enzyme.

The results can be simply interpreted on the as yet unproven assumption that the normal appearance and disappearance of tyrosinase activity during the life cycle of *Neurospora* is regulated by the inductive mechanism. The fact that tyrosinase activity has been shown to be inducible in wild-type supports this idea. Under this interpre-

tation, the constitutive formation of the enzyme during growth of the organism is the result of self-induction, the inducer or inducers originating in the intracellular pool of aromatic amino acids. The induction could be initiated by an increase in the concentration of the inducer, or by a decrease in the concentration of the repressor which is apparently derived from sulfate. The biochemical defect of *ty*-1 and of *ty*-2, according to this interpretation, could then be a deficiency of inducer or, alternatively, an excessive production of the repressor. The first of these possibilities implies a subnormal intracellular level of aromatic amino acids in the mutants, and it might be supposed that this would reveal itself as a growth requirement for aromatic amino acids. That this is not necessary, however, follows from the fact that significant induction by the L-stereoisomers—assumed to be the natural inducers—occurs only at concentrations exceeding those required for optimal growth of aromatic-requiring mutants by at least an order of magnitude. It is thus possible for the mutants to be inducer-deficient, but nutritionally normal.

In principle, our results resemble those of Pardee, Jacob, & Monod (1959), who have found that different genes control, respectively, the synthesis and inducibility of β-galactosidase in *E. coli*. Two points of difference should be mentioned, however. First, the two mutant genes we have dealt with are unlike the *i* gene of *E. coli* in that they do not determine whether enzyme synthesis will be constitutive or inductive, although superficially they seem to do so. As we have shown, mutants and wild-type alike are capable of inductive tyrosinase synthesis; but whereas induction is obligatory in the mutants, it is obligatory only on high-S medium in wild-type. On low-S medium, the synthesis in wild-type is constitutive, which we interpret as self-induction. Second, it should be noted that whereas the genes governing synthesis and inducibility in the *E. coli* system are closely linked, those in the *Neurospora* system assort independently, or nearly so.

Our findings are in accord with those of Markert (1950), who found that tyrosinase production in *Glomerella cingulata* is under multigenic control. Our data go a little farther, however, in showing that the several genes are not functionally equivalent, but can be differentiated on the basis of whether they have a structure-determining or a regulative role in tyrosinase synthesis. It is tempting to think that multigenic control of this sort may be the rule for non-essential enzymes, such as tyrosinase, that appear only in specific tissues or at specific stages of development.

Although the primary concern in this investigation was not the relation between tyrosinase and sexuality in *Neurospora*, our findings seem to make untenable the hypothesis that the female sterility of *ty*-1 and *ty*-2 is caused by their tyrosinase deficiency. The possibility that both mutants involve closely linked fertility genes appears remote, although not excluded. In view of the considerable amount of evidence favouring the view that tyrosinase is involved in perithecium development (Hirsch, 1954), it seems more likely that the female sterility and tyrosinase deficiency of the mutants will be found to be related through a common biochemical defect.

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X-ray Diffraction Studies of the Structure of Ribosomes from *Escherichia Coli*

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A study has been made of the X-ray diffraction from ribosomal particles from *Escherichia coli* and RNA extracted from the particles. At 98% relative humidity the pattern from the particles resembles that from a mixture of RNA and protein; when dry the pattern is similar to that from protein. We conclude that the RNA in the particles has a similar structure to that of extracted RNA. The diffraction pattern and the increase of ultraviolet absorption as the temperature is raised both indicate that the RNA consists largely of a double-helical structure. The nature of the hydrogen bonding in such RNA is discussed. The variation with humidity of the diffraction pattern of the particles resembles that of nucleohistone and indicates that the nucleic acid and protein in the particles have an appreciable degree of structural independence.

1. Introduction

Various X-ray diffraction studies have been made of the molecular structures of nucleoproteins, e.g. in nucleoprotamine (Feughelman *et al.*, 1955) and nucleohistone (Wilkins, Zubay & Wilson, 1959). DNA retains its double-helical structure, whereas in tobacco mosaic virus (TMV) the RNA is an extended single polynucleotide chain and its configuration is believed to be determined by attachment to protein (Franklin, Caspar & Klug, 1958). Isolated RNAs from TMV and other sources have all been found to give the same X-ray diffraction pattern although the base ratios differ. The pattern has a general resemblance to that of DNA though it differs from it (Rich & Watson, 1954) and resembles more closely (e.g. Rich, 1959*a*) that given by the copolymer of polyribouridylic and adenylic acids or by the complex of polyinosinic and cytidylic acids (Rich, 1959*b*). The base composition of RNA is not compatible with complete base-pairing of the type found in DNA. It has been suggested that RNA has a double-helical structure roughly like DNA but less regular and with fewer hydrogen bonds between bases. Strong indications that the helical structure of RNA is retained in solution have been obtained by Doty, Boedtker, Fresco, Haselkorn & Litt (1959). They found for nucleic acid and polynucleotides a correlation between the hyperchromicity (percentage increase of ultraviolet absorption on heating a solution), the optical rotation and the rate of reaction with formaldehyde, and concluded that hyperchromicity is a measure of the percentage of the nucleotides that are arranged in a helical structure. The percentages thus obtained for RNA from TMV and from liver ribosomes were 60 and 40 respectively. Indications of helical structure in TMV RNA were also obtained by Haschemeyer, Singer & Fraenkel-Conrat (1959) who studied the effect of salt concentration on the ultraviolet absorption and other physical properties of the RNA. Preliminary measurements of hyperchromicity and formaldehyde reactivity of liver ribosomes indicated 25% helical structure in the RNA

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(Hall & Doty, 1959). An X-ray diffraction study of liver and yeast ribosomes (Franklin, Klug, Finch & Holmes, 1958) led to the conclusion that the structure of RNA in the particles was different from that of extracted RNA. In the present work we describe an X-ray diffraction and hyperchromicity study of *Escherichia coli* ribosomes and RNA extracted from them. For comparison we include some observations on turnip yellow mosaic virus (TYMV).

2. Materials and Methods

1. Ribosomes were made according to the method of Tissières & Watson (1958).

2. RNA was isolated from purified ribosomes by the phenol method developed by Kirby (1956) and with modifications due to Dr. G. L. Brown.

3. The protein from the ribosomes was prepared in two ways: (1) from the interphase layer between the phenol-rich and the phenol-poor layers, as a side-product in the RNA preparation, and (2) by the method of Fraenkel-Conrat, used in the isolation of protein from TMV.

4. TYMV almost free of salt was obtained by dissolving in water material crystallised from ammonium sulphate solution, and sedimenting the TYMV in the ultracentrifuge.

5. The water content of the specimen during X-ray exposures was determined by the humidity of hydrogen passing through the X-ray camera. Saturated solutions of potassium chlorate, sodium tartrate and sodium chlorate were used to fix the relative humidity at 98, 92 and 75% respectively.

3. Experimental Results

In Plate I is shown an X-ray diffraction pattern of ribosomes from *E. coli* at 98% relative humidity (on the left), and of ribosomal RNA at 75% relative humidity (on the right). The patterns are juxtaposed so that their similarity may be seen easily. The RNA pattern contains six rings corresponding to spacings of about 25 Å, 11 Å, 7·5 Å, 5·5 Å, 4·0 Å and 3·3 Å. At 92% relative humidity the pattern is similar except that the 3·3 Å reflection is stronger and the 5·5 Å and 4 Å rings do not show clearly. For comparison with the RNA pattern we show in Plate III the partly-oriented pattern of calf thymus DNA (sodium salt) that has been denatured by boiling in water for 15 min at a concentration of 0·2%.

The pattern due to the ribosomes is less clearly defined, but rings may be seen at 29 Å, 11 Å, 7·5 Å and 3·3 Å; and there is also diffuse scattering in the region between 4 and 5·5 Å, central scattering at small angles, and a suggestion of a ring at 42 Å. We find that the X-ray diffraction patterns of ribosomes with sedimentation coefficients of 100 s, 70 s, and 50 s (Tissières & Watson, 1958) show no obvious difference. In one case a pellet which had been used for X-ray diffraction was redissolved in a solution of 5 mM-magnesium acetate + 0·5 mM-tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffer, and gave a sedimentation diagram which showed that the ribosomes had remained intact during the X-ray experiment.

The diffraction patterns of dried ribosomes and protein prepared from them are shown in Plate II. The patterns are very similar and consist of rings at about 4·5 Å and 9·7 Å and central darkening due to small-angle scattering. When dry particles are rehydrated at 98% relative humidity the pattern obtained is the same as if the particles had not been dried. Evidently the change of the pattern with water content is reversible. The pattern due to protein does not change much when the relative humidity is raised from zero to 98% but does become somewhat sharper.

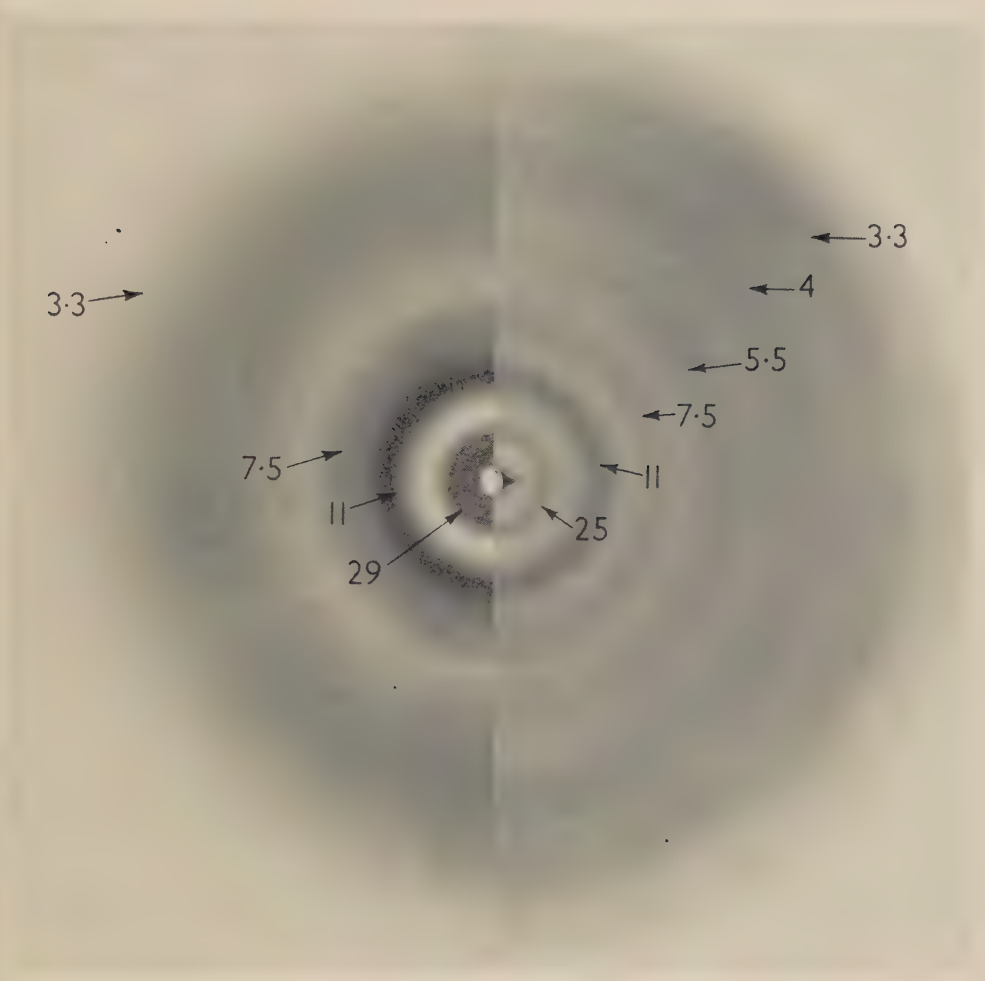

PLATE I. X-ray diffraction patterns. On the left, 50 s ribosomal particles at 98% relative humidity. On the right, ribosome RNA at 75% relative humidity. The positions of rings are shown in Å. All plates are on the same scale.

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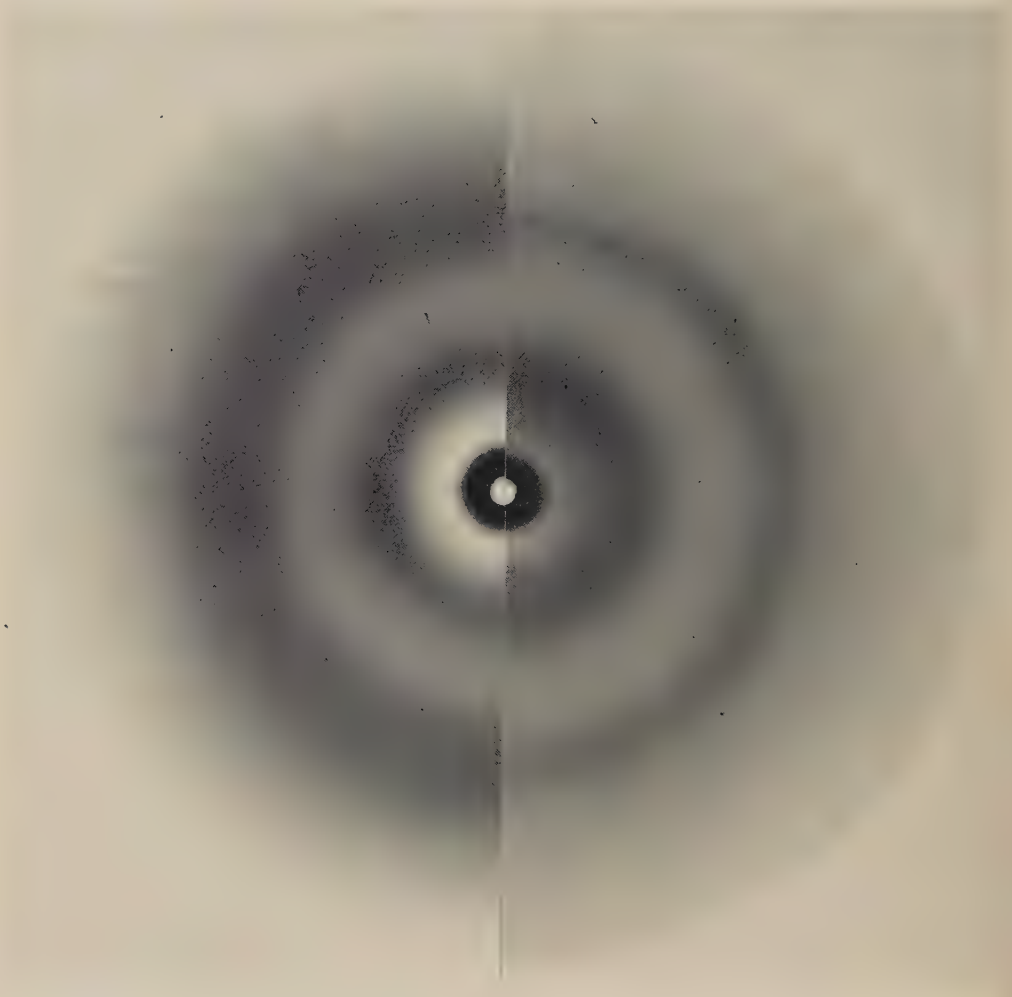

PLATE II. X-ray diffraction patterns. On the left, 50 s ribosomal particles, dry; on the right ribosomal protein, dry.

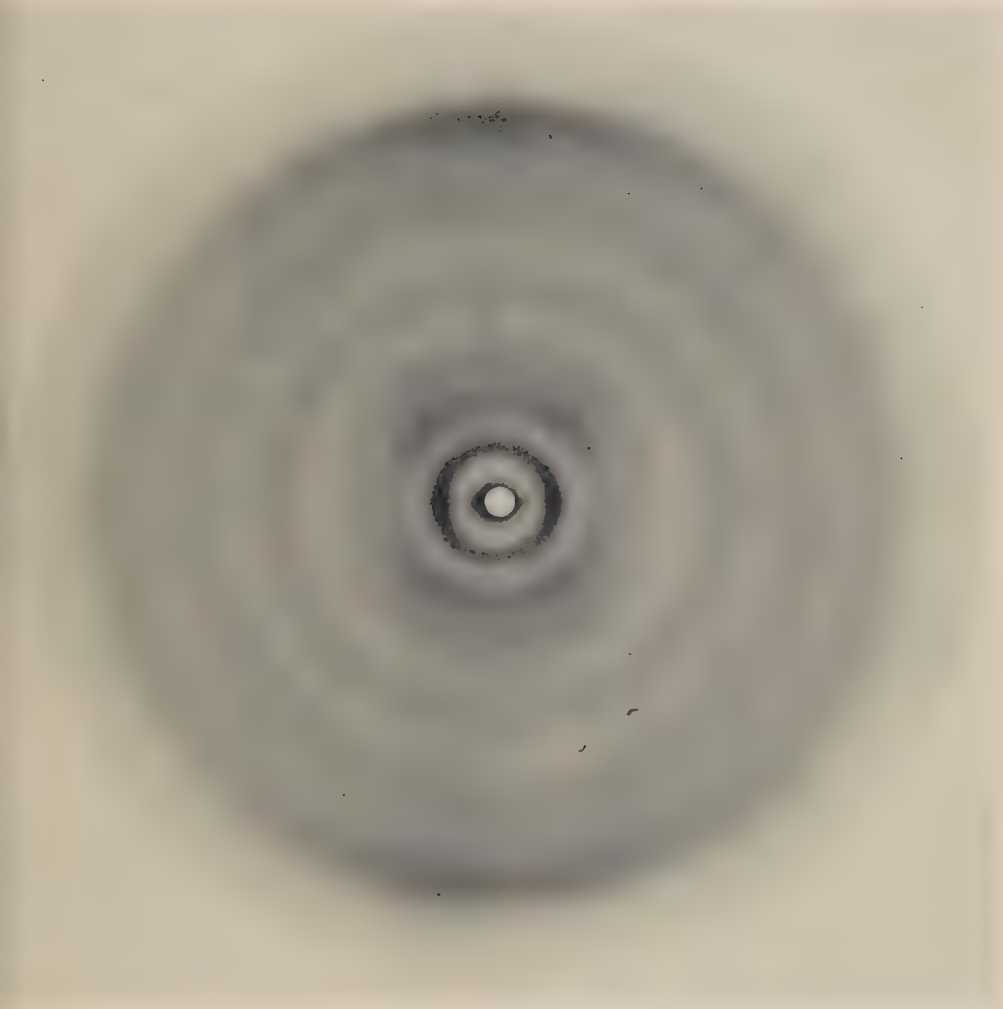

PLATE III. X-ray diffraction pattern of partly-oriented heat-denatured NaDNA at 92% relative humidity.

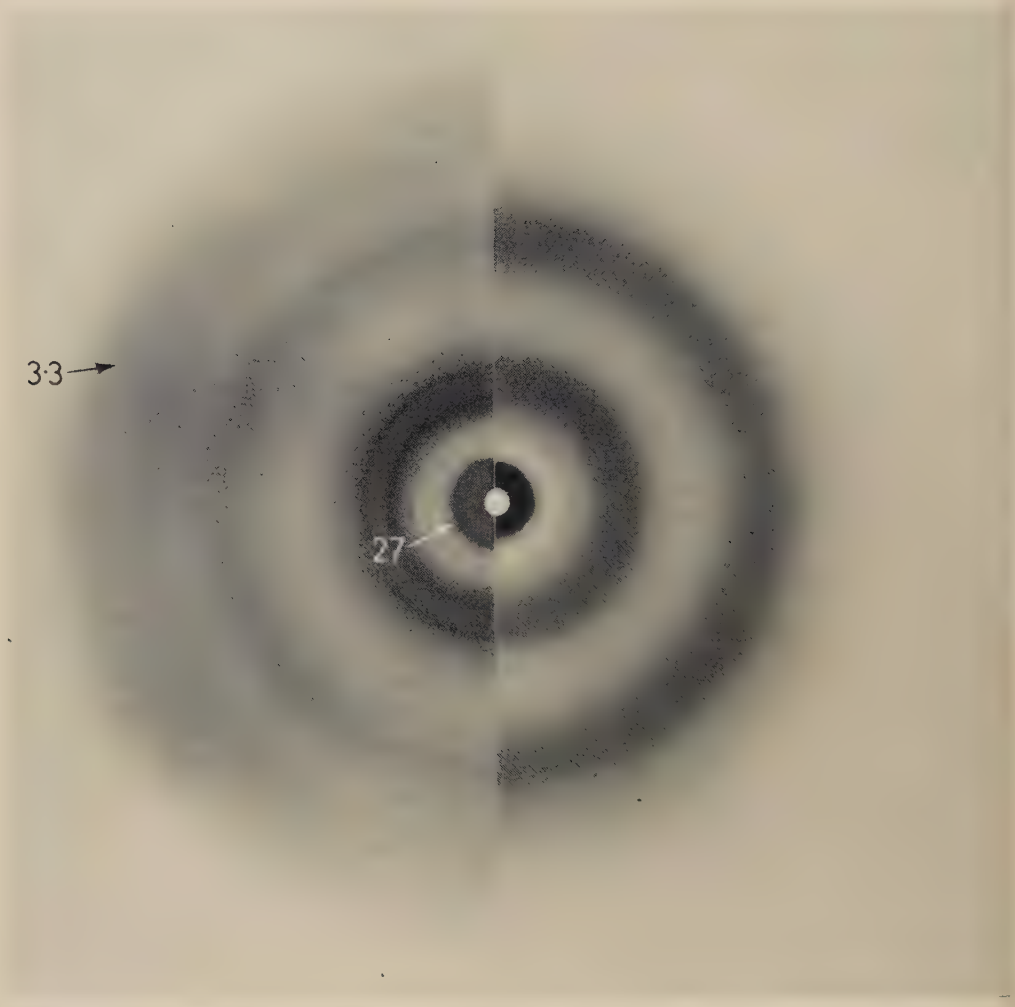

PLATE IV. X-ray diffraction patterns. On the left TYMV at 98% relative humidity; on the right, TYMV, dry. The positions of rings are shown in Å.

Fig. 1 shows the variation of optical density with temperature for ribosomal RNA in 0·1 M-phosphate buffer at pH 7·0. The optical density at 255 mμ increases 31% on heating from 25°C to 85°C. When a similar heating experiment is done on the

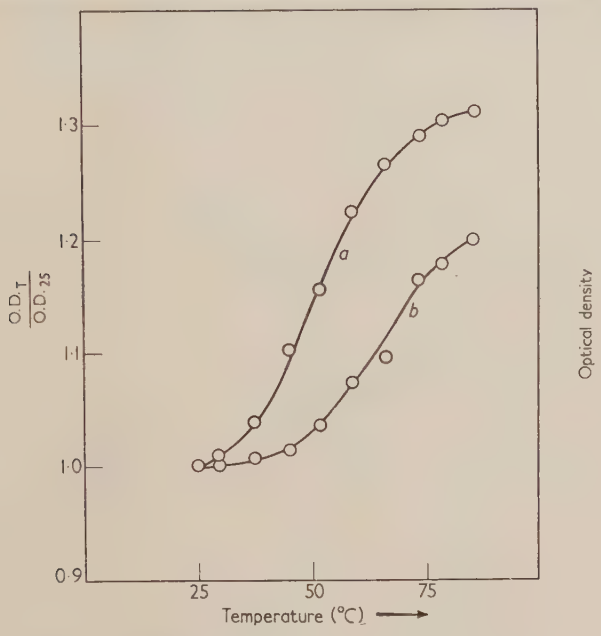

Fig. 1. Variation of optical density with temperature: *a*. microsomal RNA at 255 mμ. *b*. TYMV at 255 mμ.

ribosomes in the solvent in which they are stable (2 mM-magnesium acetate + 0·2 mM-tris buffer), no change occurs until about 60°C, at which point the optical density rises sharply and increases by 48%. Similar results have been obtained by Hall & Doty (1959) for liver ribosomes. In our experiment the optical density

Table 1

Material	Extinction coefficient at 255 mμ for solution containing 1% P
Ribosome preparation *a*	2670, 2650 } average value
Ribosome preparation *b*	2600, 2570 } 2620
RNA preparation *c*	2580, 2510, 2550, 2670 } average value 2620
RNA preparation *d*	2700, 2700

increase is at least partly due to precipitation which occurs on heating, and thus we do not obtain an exact measure of the hyperchromic effect. As an alternative approach, we have measured the extinction coefficient at 255 mμ in terms of phosphorus content of the ribosomes and of RNA extracted from them. The extinction coefficients of solutions containing 1% phosphorus are 2620 for both the nucleoprotein and the nucleic acid (Table 1). The corresponding value for TMV RNA is

2560 at 258 mμ (Haschemeyer *et al.*, 1959). It is unlikely that the protein contributes more than two or three per cent to the optical density at 255 mμ of the ribosomes. Hence we conclude that the extinction coefficient of the RNA in the ribosomes is about the same as that of the free RNA in 0·1 M-phosphate buffer. If protein absorption or light scattering contribute to the optical density of the ribosomal particles, the extinction of RNA in the particles will be less than that of free RNA. Absorption curves are shown in Fig. 2.

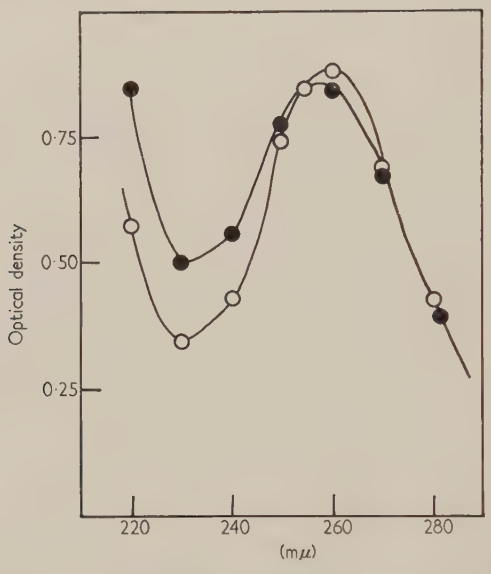

FIG. 2. Absorption curves of ribosomal RNA, ○, and ribosomes, ●.

The X-ray diffraction patterns of TYMV at 98% relative humidity, and when dry, are shown in Plate IV. The pattern at 98% humidity consists of a large number of rings due to the fine structure of the virus particles. Most of the intensity is concentrated in regions near 4·5 Å and 9·6 Å. There is also intensity in the 3·3 Å region and a ring at about 27 Å. The pattern from dry virus has diffuse protein rings at 4·5 Å and 9·6 Å. If the virus is dried for 24 hr at room temperature in dry hydrogen the sharp rings do not reappear fully at 98% relative humidity. However, most of the changes in the pattern take place between 98 and 75% relative humidity and are reversible.

The absorption of TYMV at 255 mμ increases 20% when a solution in 1 mM-phosphate buffer is heated to 85°C (Fig. 1). The change of absorption is reversible. The virus particles appear intact when viewed in the electron microscope after being dissolved in the phosphate buffer at room temperature.

4. Interpretation of Results

The diffraction pattern of the ribosomes at 98% relative humidity is almost indistinguishable from the pattern of RNA superimposed on the pattern of protein. All the rings in the RNA pattern may be seen in the ribosome pattern except the 5·5 Å and 4 Å rings, which are obscured by diffuse diffraction which corresponds to the 4·5 Å protein ring. Diffuse diffraction between the 7·5 Å and 11 Å rings corresponds to the 10 Å protein ring and central scattering resembles that from the protein. The diffraction rings at 29 Å in the pattern of the particles and 25 Å in that of the

RNA almost certainly correspond to spacings proportional to the lateral separation of RNA molecules. Since the separation of the molecules depends on hydration and binding to protein, we do not expect the spacings to be more than roughly the same in the particles and in free RNA. When the particles are dried the RNA-like rings disappear and the diffuse rings that resemble those due to protein become more intense. There seems little doubt that the scattering of the particles is the sum of the scattering from RNA and protein that behave as though they were to a large extent structurally independent. Presumably at high humidity the RNA molecules, like those of DNA, are expanded and can adopt a regular configuration which corresponds to the system of rings in the RNA pattern. When dehydrated the molecules, again like those of DNA (Wilkins, Gosling & Seeds, 1951; Franklin & Gosling, 1953), crumple into irregular shapes and the RNA pattern disappears. The protein molecules preserve their structure on drying, but when hydrated the presence of water between the molecules reduces the intensity of scattering. Hence the protein pattern is more intense at lower humidities. The behaviour of nucleohistone (Wilkins *et al.*, 1959) with respect to water content is very similar—at high humidity the diffraction pattern is largely due to nucleic acid, at low humidity to protein.

The diffraction pattern of the RNA closely resembles that of disoriented double-helical DNA in the *B* configuration. The 25 Å ring corresponds to the equatorial and first layer-line reflections of an oriented DNA *B* pattern, e.g. Fig. 5 in Wilkins (1956) and Plate III; the 11 Å ring corresponds to the strong inner part of the second layer-line, the 7·5 Å ring to the inner part of the third layer-line, the 5·5 Å ring to parts of the fifth, third, second and zero layer-lines, the 4 Å ring mainly to the eighth layer-line and the 3·3 Å ring to the tenth. The innermost ring and the 3·3 Å ring are less intense in the RNA pattern than in that of DNA. Oriented patterns have not been obtained from the RNA, yet the close correspondence of the RNA and DNA patterns makes it almost certain that a large part of the RNA, free and in the ribosomes, has a double-helical structure. One would expect that polynucleotide chains with random configuration would give a diffuse X-ray pattern readily distinguishable from a helical pattern: this effect is observed with polyadenylic acid (Fresco, 1959).

The hyperchromicity of the free RNA suggests that about thee-quarters of the RNA has a helical structure. Since the extinction coefficients of free RNA and of the RNA in the ribosomes are the same, we expect the hyperchromicity and percentage of helical structure in the two cases to be the same. It seems rather unlikely that interaction with protein would markedly affect the extinction of the RNA, although this interaction might well stabilise the RNA and cause the temperature of denaturation to be raised, as is in fact observed. Similarly the hyperchromicity of DNA is reduced when DNA is combined with histone (Zubay & Doty, 1959).

The diffraction rings given by the protein in the particles resemble the diffraction from histone (Wilkins *et al.*, 1959). It is not possible to decide, from consideration of the unoriented pattern, whether the polypeptide chains have an α or β configuration. However, the rates of deuteration of the peptide N-H groups in the particles at low and high humidities have been measured by Mr. G. R. Wilkinson. The rates are slow and this indicates that there is strong hydrogen bonding in the protein. It appears likely, therefore, that ribosomal protein, like histone (Zubay & Wilkins, unpublished), consists largely of α-helical protein.

TYMV resembles ribosomes and nucleohistone in so far as the X-ray diffraction pattern at high humidity has features that can be ascribed to nucleic acid and that disappear when the humidity is lowered. For example, the ring at 27 Å observed at

98% relative humidity is known to be due to RNA (private communication from Dr. A. Klug) and resembles the equatorial diffraction from an assembly of parallel double-helical nucleic acid molecules. The 3·3 Å diffraction at high humidity may be due to RNA but could also be due to water. The general distribution of intensity in the protein part of the TYMV pattern does not change much with humidity. It appears likely, therefore, that the protein in TYMV hydrates less than the protein in ribosomes and in nucleohistone.

The hyperchromicity of the RNA in TYMV is only 20% in the temperature range covered. The observed value indicates that about half of the RNA probably has a double-helical structure.

5. Hydrogen Bonding between Bases in Helical RNA

Something may be learned about hydrogen bonding in RNA by considering the structure of denatured DNA. The sodium salt of DNA gives a diffuse *B* type X-ray diffraction pattern (Plate III) after it has been boiled in water. The intensity of the pattern shows that a large part of the double-helical structure has re-formed after heating. We can see, however, that the helical structure has become irregular, because the *B* pattern is more diffuse after heating than before and because the denatured DNA does not crystallise in the *A* form when the relative humidity is reduced to 75%. The hyperchromicity at 260 mμ of heat-denatured DNA is 30% in 0·14 M-NaCl + 0·14 M-sodium citrate. This indicates that about three-quarters of the DNA has a helical structure. We assume that all the hydrogen bonds in DNA are broken in water at 100°C. It is not likely that the polynucleotide chains can so arrange themselves on cooling that all the Watson-Crick base pairs can re-form. Our experimental results and many other previous ones, e.g. those of Laland, Lee, Overend & Peacocke (1954) show that they do not. One would expect (e.g. Doty *et al.*, 1959) that many types of base pairs (Donohue, 1956) exist in denatured DNA. The main pairs would probably be the Watson-Crick pairs—guanine-cytosine with 3 hydrogen bonds and adenine-uracil with 2 bonds, cytosine-uracil with 2 bonds (pairing 7 in Donohue, 1956), adenine-guanine with 2 bonds (pairing 22 in Donohue, 1956). All these pairs have hydrogen bonds between 6-keto and 6-amino groups and are likely to predominate when the number of these groups in nucleic acids is equal as it is in DNA and in many RNAs (Elson & Chargaff, 1954) including *E. coli* RNA (Horowitz, Lombard & Chargaff, 1958). When a pair of bases do not match suitably, only one hydrogen bond or none might be formed. The percentage of helical RNA structure in TYMV is lower than in *E. coli* ribosomes; this may be due to the low ratio, 0·65 (Markham & Smith, 1951), of the number of 6-keto to 6-amino groups in TYMV.

The distance between glycosidic bonds in a cytosine-uracil pair is about 2·5 Å less than in Watson-Crick pairs and the distance in adenine-guanine is 2 Å greater. The direction of the glycosidic bonds is the same in all pairs but because of the different distances between pairs of bonds the distance of the phosphate-ester chain from the helix axis in a helical molecule will vary by about 1 Å. The molecule will therefore be somewhat irregular. Since the chain in DNA can adopt three different configurations—the *A*, *B* and *C* forms (Marvin, Spencer, Wilkins & Hamilton, 1958)—there appears to be no *a priori* reason why an irregular configuration should not be stereochemically possible. Apart from the hydrogen bonding, the structure will be stabilised by forces, due to water, that tend to make the bases stack on each other. Water will hydrogen bond roughly in the plane of the bases; therefore, perpendicular to the

plane, there will be a 'hydrophobic attraction' between bases when they are placed in water. The energy involved is probably about equal to that of the hydrogen bonding between bases.

When such a double-helical structure is formed from ribose chains it will be somewhat different from the DNA structure because the hydroxyl group of the ribose cannot be accommodated in the *B* configuration of DNA. The modification in the structure need not be large and the existence of DNA-like complexes of polyribouridylic and adenylic acids (Rich 1959*a*, *b*) shows that such a structure can be formed.

In structures of the type we have discussed, the sequence of atoms in the phosphate-ester chain is opposite in direction in the two chains. In double-helical polyriboadenylic acid (Rich 1959*a*) the sequences are believed to be in the same direction. X-ray diffraction analysis does not provide a direct means of distinguishing between double-helical molecules that consist of two strands or of one strand folded back on itself. However, one would expect that single-stranded double-helical molecules would form most readily if the sequence of atoms in the two chains were opposite in direction. Therefore if the diffraction pattern resembles that of the structure in which the directions are the same, this may be taken as evidence that the molecules are probably not single-stranded. The diffraction patterns of RNA closely resemble the DNA pattern superimposed on the polyadenylic acid pattern. It seems possible that RNA that gives the type of X-ray pattern published (Rich & Watson, 1954; Rich, 1959*a*, *b*) is a mixture of slightly irregular structures resembling those of DNA and polyadenylic acid, and also contains non-helical regions where the bases do not match sufficiently well to form a hydrogen-bonded double-helix. The evidence does not enable us to decide whether the molecules are single- or double-stranded. Because we have not been able to obtain an oriented diffraction pattern from the ribosomal RNA, it is not clear whether the RNA consists of a mixture of DNA-like and poly-A-like structures or is predominantly like DNA. Unpublished X-ray data (obtained by G. Zubay & G. L. Brown) indicate that *E. coli* 'soluble' RNA has a structure like DNA. With regard to the function of the RNA we may note that RNA need not have a well-defined helical structure if it is to function as a template: if an enzyme is involved in this function, the enzyme may arrange the RNA in the desired configuration.

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Nucleotide Composition of Soluble Ribonucleic Acid from *Escherichia coli*

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The proportions of the four main nucleotides and a number of additional components in soluble-RNA from *Escherichia coli* have been determined. Pseudouridine and thymine were found to be present in comparatively high proportions and smaller amounts of 2-methyladenine, 6-methylaminopurine and 1-methylguanine were detected. No evidence was found for the presence of a number of other methylated bases or 2-O-methylribose and it was shown that the 6-methylaminopurine did not arise from 1-methyladenine residues during the hydrolysis of the RNA. The terminal nucleoside residues appear to be almost exclusively adenosine and the proportion agreed with the molecular weight of 25,000 reported for this RNA.

The preparation and some of the properties of soluble ribonucleic acid (S-RNA) from *Escherichia coli* have been described (Tissières, 1959). We have determined the proportions of the four principal nucleotides in this material using ion-exchange chromatography. As the S-RNA from rat liver has been found to contain comparatively high proportions of a number of additional components (Dunn, 1959) we have also examined the S-RNA from *E. coli* for these minor components using paper chromatography and electrophoresis.

For the ion-exchange chromatography three different preparations were hydrolysed in 0·3 M-KOH and the alkali was neutralized with perchloric acid according to Smith (1955). Normally 18 hr at 37°C was used for the hydrolysis but the third preparation was incubated for 36 hr in order to ascertain whether digestion was complete after the shorter time. The third preparation of RNA was also hydrolysed with a ribonuclease isolated from the ribonucleoprotein particles of *E. coli* which gives rise to the 3′-phosphates of both purine and pyrimidine nucleosides (Spahr & Hollingworth, 1960). The RNA was incubated with the enzyme in 0·1 M-tris buffer pH 7·4 containing 0·05 M-NaCl for 48 hr at 37°C. The nucleotide mixture obtained from alkali or ribonuclease hydrolysis was diluted and chromatographed on Dowex CG 1×8 by the procedure of Cohn (1950) under the conditions described previously (Spahr & Tissières, 1959).

In the four analyses the recoveries from the column represented 98 ± 2% of the added optical densities. The four analyses gave very similar results, the peaks corresponding to the four principal nucleotides representing about 87% of the material while the remainder was distributed among a number of minor peaks. Three of these, representing a further 3% of the added optical densities, have been shown to correspond with adenosine, pseudouridine 2′-phosphate and pseudouridine 3′-phosphate by

chromatographing these compounds on the column together with synthetic nucleotide mixtures under conditions identical with those used for the hydrolysates. Several of the other minor peaks may well represent the other additional components described below. About 5% of the material from the hydrolysate was present, however, in a single peak which was eluted from the column with N-HCl but not with N-formic acid. In the third analysis this material was further resolved into seven peaks by gradient elution using formic acid and ammonium formate. The amount and spectrum of the first of these peaks suggests that it is thymine ribonucleotide, but the remaining peaks (representing about 4% of the total optical density) probably do not correspond with any of the components described below. Some of the material may be guanosine diphosphate which has recently been shown to be present in alkaline hydrolysates of S-RNA from *E. coli* and rabbit liver (Zillig, Schachtschabel & Krone, 1960; Singer & Cantoni, 1960), but other compounds must also be present in this fraction. From the position in which they are eluted from the column they might be a residue of oligonucleotides which are more resistant to the alkaline hydrolysis. The presence of such compounds in alkaline hydrolysates of RNA has been described by Lane & Butler (1959). The proportion of these components did not, however, appear to be reduced by the increased time of hydrolysis and, as described below, we found no evidence for the presence in this RNA of the alkali-stable dinucleotides detected in some other ribonucleic acids (Smith & Dunn, 1959*b*).

TABLE 1

Nucleotide composition of S-RNA

	mol/100 mols identified nucleotides
Adenylic acid†	20·3 ± 0·5‡
Guanylic acid	32·1 ± 0·1
Cytidylic acid	28·9 ± 0·3
Uridylic acid	15·0 ± 0·4
Pseudouridylic acid	2·1
Thymine ribonucleotide	1·1
2-Methyladenylic acid	0·3
6-Methylaminopurine ribonucleotide	0·1
1-Methylguanylic acid	0·1
Ratios	
Purines/pyrimidines	1·12
6-amino/6-keto	0·98

† Value includes terminal adenosine.

‡ Errors are expressed as standard deviations from the mean of four estimations.

The mean values for the proportions of the four principal nucleotides obtained from the four analyses have been combined in Table 1 with estimates of the minor components made relative to uridylic acid as described below. The results are relative to the identified components, no allowance being made for the unidentified material described above. Similar results for the proportions of the four main nucleotides in

this type of RNA have also been obtained by Bolton (1959) and Dr. P. Berg (personal communication).

To examine this S-RNA for the additional components, the RNA was hydrolysed to mononucleotides with 1 N-KOH at 28° C for 18 hr and the hydrolysate was examined for the presence of thymine, methylated adenines, methylated guanines, 5-methylcytosine, and pseudouridine by the methods previously described (Littlefield & Dunn, 1958; Smith & Dunn, 1959*a*; Dunn, 1959, 1960). Only thymine, 2-methyladenine, 6-methylaminopurine, 1-methylguanine and pseudouridine were detected and these were estimated relative to the uridylic acid in the hydrolysate (Table 2). If the other methylated bases had represented more than 0·1 mol/100 mol uridylic

TABLE 2

Proportions of additional components in S-RNA from E. coli

Component	mol/100 mols uridylic acid	
	KOH hydrolysis	HCl hydrolysis
Pseudouridine	14	—
Thymine	7	8
2-Methyladenine	1·7	1·6†
6-Methylaminopurine	0·6	0·6†
1-Methylguanine	0·9	—

The following components were not detected in this RNA (more than 0·1 mol/100 mols uridylic acid would have been detected): 5-methylcytosine, 6-dimethylaminopurine, 1-methyladenine, 2-methylamino-6-hydroxypurine, 2-dimethylamino-6-hydroxypurine. No evidence was obtained for the presence of dinucleotides containing 2-O-methylribose.

† Molar extinction coefficients in 0·1 N-HCl for 2-methyladenine and 6-methylaminopurine were respectively those of Baddiley, Lythgoe & Todd (1944) and Dunn & Smith (1958).

acid they would have been detected. The dinucleotides containing 2-O-methylribose (Smith & Dunn, 1959*b*) present in the S-RNA from rat liver were also not detected in this RNA.

Studies by Brookes & Lawley (1960) have shown that the ribonucleotide of 1-methyladenine undergoes a transformation to the corresponding derivatives of 6-methylaminopurine in mild alkaline conditions. This work makes it necessary to establish whether 6-methylaminopurine or 1-methyladenine residues are originally present in nucleic acid, when 6-methylaminopurine is detected after the material has been subjected to alkaline conditions. Studies by Dunn & Smith (unpublished) indicate that 1-methyladenine is the compound present in pig-liver S-RNA, while 6-methylaminopurine is present in the DNA of *E. coli*. A sample of the S-RNA from *E. coli* was therefore hydrolysed in 1 N-HCl at 100°C for 1 hr (Smith & Markham, 1950). The products were separated by paper chromatography in *iso*propanol-water-HCl (Wyatt, 1951) and the adenine band examined for 1-methyladenine by successive chromatography in *iso*propanol-water-NH_3 (Markham & Smith, 1952) and *n*-butanol-water-H.COOH (Markham & Smith, 1949). No detectable amount of 1-methyladenine was found. In agreement with this, 6-methylaminopurine and 2-methyladenine were detected in the region of the *iso*propanol-water-HCl chromatogram between adenine and cytidylic acid after rechromatographing this material in

*iso*propanol-water-NH_3. The proportions present corresponded closely to those estimated from the alkaline hydrolysate (Table 2). The material on the *iso*propanol-water-HCl chromatogram in the region of the cytidylic and uridylic acids was re-chromatographed in *iso*propanol-water-NH_3 and the nucleotides were examined for thymine, 5-methylcytosine and pseudouridine by the methods applied to the alkaline hydrolysate. Thymine but no 5-methylcytosine was detected (Table 2). The amount of pseudouridine recovered was only about 65% of that found in the alkaline hydrolysate. This was expected from the studies of Yu & Allen (1959) and Cohn (1959) which indicate that pseudouridine and its nucleotide are partly converted by acid hydrolysis to other compounds with different chromatographic properties. The bases in the acid hydrolysate were estimated relative to the amount of uridylic acid plus uridine present.

In addition to the nucleotides, the alkaline hydrolysate contained a small proportion of nucleosides which presumably arose from terminal nucleoside residues. They were separated from the mononucleotides by chromatography in *iso*propanol-water-NH_3 and identified spectroscopically after chromatography in *n*-butanol-water-H.COOH and *n*-butanol-water-NH_3 (Markham & Smith, 1949). In agreement with the studies of Preiss, Berg, Ofengand, Bergman & Dieckmann (1959), adenosine was found to represent over 90% of the material present. The proportion of adenosine determined in this manner was in close agreement with the proportion determined from the ion-exchange chromatograms, and indicated that there was one adenosine end-group for about 80 nucleotides. This is in agreement with the molecular weight of 25,000 determined for this RNA (Tissières, 1959) but not with that obtained by Brown, Brown & Gordon (1959) for S-RNA they prepared from *E. coli*.

The proportions of the main nucleotides present in S-RNA from *E. coli* (Table 1) are considerably different from those in RNA from ribonucleoprotein particles (Spahr & Tissières, 1959). The biggest differences are found with respect to cytidylic acid which is over 35% higher and uridylic acid which is 40% lower in S-RNA than in particulate RNA. A further difference is that while the 70 s particles have only been found to contain a small amount of pseudouridine (0·8 mol/100 mols uridylic acid) (Dunn & Smith, unpublished) the S-RNA from *E. coli* contains comparatively high proportions of several additional components. In this it resembles the S-RNA from rat liver (Dunn, 1959) and yeast (Otaka, Hotta & Osawa, 1959; Davis, Carlucci & Roubein, 1959), but it differs from these in the additional components present and in their proportions. The only additional components detected in all these three types of S-RNA have been pseudouridine and 1-methylguanine. While 6-methylaminopurine has been identified in alkaline hydrolysates of all three types of RNA, our studies on pig-liver RNA and an unpublished observation by Dr. K. S. Kirby suggest that 1-methyladenine is the component present in the rat-liver RNA.

While the S-RNA from *E. coli* has been shown to contain thymine rather than 5-methylcytosine, the latter compound has been isolated from the S-RNA of another bacterium (Dr. H. Amos, personal communication). Further work will be necessary to establish whether the thymine residues arise from 5-methylcytosine residues during the isolation of the RNA as has been suggested (Dunn, 1960).

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Electron Spin Resonance of Copper Proteins and Some Model Complexes

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Electron spin resonance spectra of a number of copper proteins (laccase, ceruloplasmin, erythrocuprein and Cu^{2+}-carboxypeptidase) and of some small-molecule complexes, with known coordinating atoms, have been recorded, mainly in frozen aqueous solutions. The method used in evaluating the spectra has been discussed, and the g-values and the hyperfine structure constants as well as the absorption maxima in the visible spectra have been determined. A qualitative theory for correlating the experimental data with the bonding of the Cu(II) ion has been given. An observed trend in g-values and hyperfine structure constants suggests that the metal is coordinated to N in the proteins. The metal in the oxidative enzymes (laccase, ceruloplasmin) appears coordinated in a unique manner, as evidenced by their exceptionally low hyperfine structure constants. This can be related to a high degree of delocalization of the unpaired electron. The unique bonding disappears on denaturation, and its possible relation to the oxidase activity of the proteins has been discussed.

1. Introduction

The electron spin resonance (ESR) technique (see, for example, Ingram, 1955; Sogo & Tolbert, 1957) offers a powerful tool for studying the interaction of transition-metal ions with their chemical environment. We have here explored the use of ESR measurements in our investigations on the role of metals in enzymic catalysis. Previous reports (Malmström, Mosbach & Vänngård, 1959; Bray, Malmström & Vänngård, 1959) have been concerned with the valence state of the metal and its change during catalytic action. These studies have now been extended in an attempt to obtain clues to the mode of binding of the metal to the protein, and in the present report we will describe ESR studies on a number of copper proteins. To get a basis for interpreting the results, we have also investigated several model complexes having the same coordinating atoms as present in proteins.

2. Materials and Methods

(a) *Proteins*

Fungal laccase was prepared as previously described (Malmström, Fåhraeus & Mosbach, 1958). Ceruloplasmin was obtained from AB. Kabi, Stockholm (we are indebted to Drs. H. Gedin and L. Broman for supplying this enzyme and checking its purity). Two different preparations of erythrocuprein were used; one was prepared by the method of Markowitz, Cartwright & Wintrobe (1959) (kindly supplied by Dr. G. Shields, Salt Lake City) and the other by the method of Nyman (to be published). Thrice crystallized carboxypeptidase was obtained from Worthington Corp., Freehold, N.J.; Zn^{2+} was removed by dialysis against 1,10-phenanthroline as described by Vallee, Rupley, Coombs & Neurath (1958) and Cu^{2+} was introduced by a dialysis procedure (Nylander, to be published). All proteins were

studied as amorphous frozen-dried samples as well as in aqueous solution (pH about 5·5); the protein concentrations in solution were adjusted to correspond to a Cu^{2+} concentration of about 10^{-3} M.

(b) *Other complexes*

In addition to $Cu(NO_3)_2$, complexes of the following organic ligands were investigated: oxalic acid (I), citric acid (II), salicylic acid (III), ethylenediaminetetra-acetic acid (EDTA), 2,2′-dipyridyl (IV), 1,10-phenanthroline (V), glycine (VI), L-histidine (VII) and imidazole (VIII). Commercial reagent-grade chemicals were employed in all cases without recrystallization, and deionized water was used for making the solutions. The complexes of I-III and EDTA were made by mixing $Cu(NO_3)_2$ or $CuCl_2$ and the Na salt of the ligand in a final concentration of 0·005 M and 0·025 M, respectively. Solutions of the other complexes were prepared by titrations of the acid form of the ligands in 0·05 M concentration (except in the case of VIII, which had a concentration of 0·126 M), in the presence of 0·005 M-$Cu(NO_3)_2$, with 0·2 N-NaOH to a suitable pH, determined with a glass electrode at 22° C. The following pH values were chosen with the different ligands: IV, V and VIII 6·8; VI 8·2; and VII 7·8. The concentrations of complexes present can be calculated by the method of Bjerrum, and titration data available in the literature (see Bjerrum, Schwarzenbach & Sillén, 1957) predict the 2 : 1 complexes to be the dominating species in all cases except with II and EDTA, which form 1 : 1 complexes only, and with VIII, which forms a 4 : 1 complex under the conditions used. Solutions titrated to intermediate points could not be studied, since under such conditions mixtures of several complex species are present and no well-defined ESR spectra can be obtained. It is important that the ligand is present in excess in all cases, since a change in the degree of coordination may otherwise occur during the freezing of the solutions (see *ESR technique*).

The 4 : 1 complex of VIII was crystallized by slow evaporation of the solution at 4°C. The crystals were analyzed for Cu and N (these analyses were performed by Dr A. Bengtsson in the Department of Analytical Chemistry, University of Uppsala). The values found were 13·63% Cu and 30·28% N; the calculated values are 13·82 and 30·46%, respectively, for $Cu^{2+}(imidazole)_4(NO_3^-)_2$ (this complex is designated $Cu(Im)_4^{2+}$ in the remainder of this paper). Dilute crystals of this complex, with Pd^{2+} as the other metal, were also prepared by slow evaporation at 4°C of a solution containing 0·01 M-$PdCl_2$, 0·0001 M-$Cu(NO_3)_2$ and 0·12 M-VIII (as nitrate), titrated to pH 6·7.

Visible spectra of the complexes as well as of the copper proteins were recorded in a Beckman DU spectrophotometer with 1 cm cells.

(c) *ESR Technique*

The fundamentals of ESR have been described in many places (see, for example, Ingram, 1955, and for a review of ESR applications to biology, Sogo & Tolbert, 1957). In an ESR experiment, a paramagnetic sample is placed in a static magnetic field, and the splitting by the field of the lowest electronic state is determined by applying an oscillating field to the sample. The parameters of principal interest, obtained by measuring the static magnetic field strength and the microwave frequency, are the g-values and the hyperfine constants. The g-values are a measure of the interaction between the applied magnetic field and the unpaired electrons. For a free electron $g = 2{\cdot}0023$, and departures from this value depend on contributions from the orbital motions. The hyperfine constants (usually designated A and B) show the coupling between the unpaired electrons and the nucleus.

For complexes with tetragonal symmetry, the spectra are defined by specifying the g-value and hyperfine constant with the magnetic field parallel to the symmetry axis ($g_\parallel$, A) and with the magnetic field perpendicular to this axis ($g_\perp$, B). The method used for evaluating the spectra of the frozen solutions is similar to that described by Sands (1955) and by Faber & Rogers (1959). Almost all the low-temperature spectra recorded have essentially the shape shown in Fig. 1, which is compatible with tetragonal symmetry of the complex. $g_\parallel$ and A are measured as indicated in the figure. This procedure was tested by comparing the spectra of a diluted single crystal of $Cu(NH_4)_2(SO_4)_2.6H_2O$ with the spectrum obtained when the crystal was crushed to a fine powder. $g_\perp$ is not so well defined in the spectra, and instead we have measured the g-value at maximum absorption (g_m),

which should be close to $g_\perp$. Hyperfine splitting centred around $g_\perp$ has in no case been observed (*cf.* Faber & Rogers, 1959).

The experiments were performed with a Varian V-4500 spectrometer operating at a frequency close to 9500 Mc/sec. The samples were kept in quartz tubes with 1 mm bore for room temperature work and with 3 mm bore for work at 100°K.

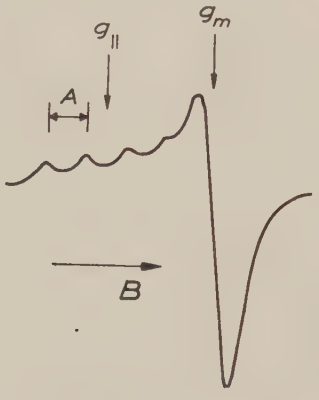

FIG. 1. Idealized ESR spectrum of frozen solutions of Cu^{2+} complexes, showing the method used in measuring the g- and A-values.

3. Results

Since all ESR spectra recorded under given conditions have the same general appearance, only a few typical absorption curves will be given. Fig. 2 shows the ESR spectrum of a frozen solution of ceruloplasmin. This gave identical spectra when studied as frozen solution, frozen-dried sample or solution at room temperature, and

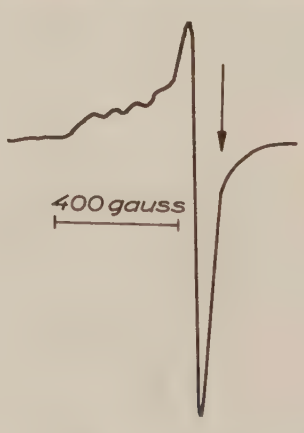

FIG. 2. ESR spectrum of a frozen solution of ceruloplasmin. The arrow in this and in the following figure indicates the resonance field for free electrons.

the same was found with the other proteins. The small-molecule complexes, on the other hand, show marked differences in the spectra recorded at room temperature and in frozen solution, as illustrated in Fig. 3. A polycrystalline sample of $Cu(Im)_4^{2+}$, in which the crystals were diluted with $Pd(Im)_4^{2+}$, yielded the same spectrum as the frozen solution, except that additional hyperfine structure (from N) was also observed.

The values of g_m, $g_\parallel$ and A have been calculated from the spectra of the frozen solutions, as described under *ESR technique*, for all complexes that gave a well-defined hyperfine structure; the uncertainty in the g-values was estimated to $\pm$ 0·005. The results are summarized in Table 1, which also gives values for the absorption maximum in the visible spectrum and for the parameters a^2 and

Table 1

Parameters characterizing the ESR and visible spectra of some copper proteins and model complexes

Ligand	Co-ordinating atoms	g_m	$g_\parallel$	$\lvert A \rvert$ (cm^{-1})	Abs. max. (cm^{-1})	a^2	$\frac{4}{7}a'^2 + \kappa$
Dithiocarbamate†	4 S	< 2·047	< 2·137	—	23000	< 0·48	—
Phthalocyanine‡	4 N	2·045 ($g_\perp$)	2·165	0·022	—	—	0·81
Laccase	—	2·048	2·197	0·009	16400	0·49	0·48
Ceruloplasmin	—	2·056	2·209	0·008	16500	0·52	0·45
Denatured laccase	—	2·055	2·23	0·020	—	—	0·83
Denatured ceruloplasmin	—	2·056	2·257	0·018	—	—	0·81
Cu^{2+}-carboxypeptidase	—	2·060	2·24	0·019	—	—	0·81
Erythrocuprein	—	2·063	2·265	0·016	15200	0·61	0·75
Bis-salicylaldehyde-imine‡	2 O, 2 N	2·045 ($g_\perp$)	2·200	0·0185	16300	0·49	0·75
Bis-acetylacetonate‡	4 O	2·053 ($g_\perp$)	2·266	0·0160	15000	0·60	0·75
Histidine	4 N	2·063	2·230	0·018	15600	0·54	0·78
Imidazole	4 N	2·063	2·267	0·018	16800	0·68	0·81
2,2′-Dipyridyl	4 N	2·082	2·27	0·017	14900	0·61	0·79
1,10-Phenanthroline	4 N	2·088	2·28	0·015	15200	0·64	0·74
Oxalate	4 O	2·078	2·316	0·017	15400	0·73	0·84
EDTA	4 O, 2 N	2·090	2·337	0·015	13900	0·71	0·80
Citrate	2 O	2·074	2·349	0·015	13700	0·72	0·82
1 N-$NaNO_3$	—	2·083	2·397	0·015	—	—	0·86

† Vänngård & Åkerström (1959).

‡ Gibson, Ingram & Schonland (1958); K. Oohata & M. Aoyagi (unpublished, Progress Report no. 7 of the Research Group for the Study of Molecular Structure, University of Tokyo) suggest that the unpaired hole is in a π-orbital rather than in a σ-orbital.

§ Maki & McGarvey (1958 *a* and *b*). The values of a^2 and $(4/7\ a'^2 + \kappa)$ given here differ from those of Maki & McGarvey, who used formulae including higher terms.

$(4/7\ a'^2 + \kappa)$, calculated as described below. The ligands have, on the whole, been arranged in order of increasing g-values. In addition to our own results, the table also includes some data from the literature.

4. Discussion

The spectra of the frozen solutions show the asymmetry typical of copper complexes. The room temperature spectra of the small molecule complexes (Fig. 3), on the other hand, are more symmetrical, since the tumbling of the molecules in solution causes an averaging of the anisotropy. The averaging is, however, only effective when the relaxation time (τ) of the rotation is comparable to or smaller than the inverse value of the anisotropy, expressed as frequency. At room temperature in aqueous solution the protein molecules have $\tau \simeq 10^{-7}$ sec (Edsall, 1953), while the inverse anisotropy

is of the order of 10^{-9} sec, so there is no averaging of the protein anisotropy. A comparison between copper proteins and small molecule complexes can, therefore, only be made with the frozen solutions, which also has the advantage of a higher sensitivity of the ESR measurements.

The aim of the present investigation was to get some idea of the bonding of the Cu(II) ion in the proteins by comparing their ESR and visible spectra between themselves and with the spectra of smaller model complexes. In view of the rather incomplete results obtained, the theory correlating the ESR and the visible spectra

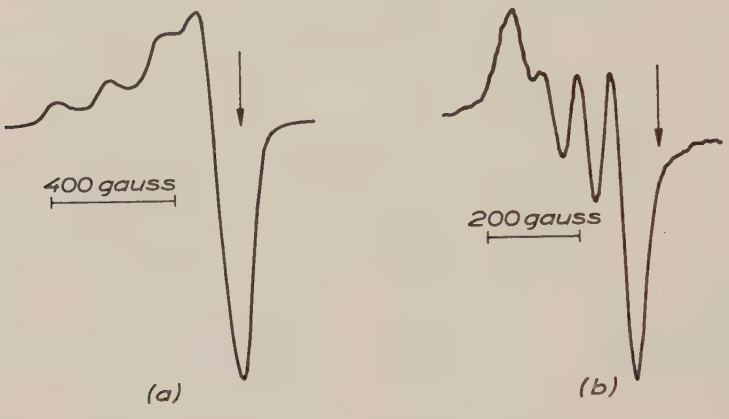

FIG. 3. ESR spectra of an aqueous solution of a Cu^{2+}-histidine complex; (a) frozen, (b) room temperature.

with the bonding parameters will be described in a qualitative way only. The g-values are given by the following approximate expressions:

$$g_{\parallel} = 2{\cdot}0023 - \frac{8\lambda a^2}{\Delta_1};$$

$$g_{\perp} = 2{\cdot}0023 - \frac{2\lambda b^2}{\Delta_2},$$

in which λ is the spin-orbit coupling of the free Cu(II) ion ($= -828$ cm^{-1}) and Δ is the energy difference between the ground state and excited electronic levels. The coefficients a^2 and b^2 give a measure of the degree of covalency of the bonding. A purely ionic complex has $a^2 = b^2 = 1$, but the values decrease with increasing covalency. The quantitative interpretation of a^2 and b^2 depends on the model used as a basis for the theory. For a complex with almost cubic symmetry, a^2 has been correlated with the covalency of the σ-bonds (Owen, 1955), while, for a square planar complex, Maki & McGarvey (1958*a*) have shown that covalency in both σ- and π-bonds in the plane of the ligands reduce the value of a^2 to much the same degree. The hyperfine structure constant A is approximately:

$$A = P[-\tfrac{4}{7}a'^2 - \kappa + (g_{\parallel} - 2) + \tfrac{3}{7}(g_{\perp} - 2)].$$

For Cu^{2+} we can use $P = 0{\cdot}035$ cm^{-1}; a'^2 is again a constant describing the covalency of the complex. Maki & McGarvey (1958*a*) relate a'^2 to the in-plane σ-bonding. The term κ comes from the isotropic Fermi electron-nuclear interaction.

Before turning to the experimental results, we want to stress the necessarily approximate nature of our analysis. We have assumed tetragonal symmetry and also neglected some terms in the expression for $g_{\parallel}$, $g_{\perp}$ and A. The assumption that the maximum absorption of visible light occurs at the same wavelength in the room

temperature solutions and in the frozen solutions at 100°K appears very questionable, and we can only hope that possible differences in the absorption maxima do not change the general conclusions drawn. Finally, we have made the reasonable assumption that the absorption of the copper proteins is due to the Cu(II) ion, but this does not affect the discussion of the hyperfine structure constants.

The preceding comments make it clear that our data do not allow any rigorous quantitative description of the bonding. A few general remarks can, however, be made. The spectra have the shape of Fig. 1, which is compatible with the assumption of tetragonal symmetry. In order to facilitate a comparison between the different complexes, we have calculated a^2 and $(4/7\ a'^2 + \kappa)$ (assuming $A < 0$) and included the values in Table 1. The a^2 values are usually smaller for complexes with N than with O, which conforms with the known tendency of N to form strong covalent complexes. The proteins have such low a^2 values that it seems likely that the coordinating atoms are N, though S may not be excluded.

The most striking result in Table 1 is the hyperfine constants obtained for the two oxidative copper enzymes, laccase and ceruloplasmin. The native enzymes have A-values considerably smaller than the other complexes, but when they are denatured, the A-values increase to those found for the other complexes, including two other copper proteins. The difference between the two oxidative enzymes and the other molecules is still more apparent from the values of $(4/7\ a'^2 + \kappa)$ (see Table 1). Apart from laccase and ceruloplasmin, this expression is rather constant with a mean value around 0·80, which could indicate that the differences between the complexes is to be found in the π-bonding rather than in the σ-bonding. The values of $(4/7\ a'^2 + \kappa)$ for laccase and ceruloplasmin, on the other hand, are 0·45 to 0·50. This could mean that the model used here completely fails for these complexes, but a more attractive interpretation would be that it is related to the oxidase activity of these enzymes. It has been shown that copper in both laccase and ceruloplasmin changes valency when the enzymes perform their catalytic action (Malmström *et al.*, 1959; Gedin, Malmström & Vänngård, to be published), and a high degree of delocalization of the unpaired hole on Cu^{2+} would facilitate such a mechanism.

Delocalization of the electron occurs also in the other complexes as evidenced, for example, by the hyperfine structure from N in the crystals of $Cu(Im)_4^{2+}$, but it is of a much lower order as shown by the values of a^2 and $(4/7\ a'^2 + \kappa)$. We have, in fact, failed to find any data in the literature indicating the same degree of delocalization in any other Cu^{2+} complexes.

The interpretation that the unique coordination of Cu^{2+} in laccase and ceruloplasmin is related to their enzymic activity is strengthened by the facts that the non-oxidase proteins fall in the same category as the small-molecule complexes and also that the oxidases are transferred to this group on denaturation, which destroys their catalytic activity. This importance of the secondary and/or tertiary structure is particularly interesting, since we have also found that the specific binding sites for catalytically active metal ions is destroyed in some other metal enzymes when the enzyme is denatured by urea (Malmström, to be published), and a detailed study of this question is being planned.

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